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MONOGRAPHS ON BIOCHEMISTRY

EDITED BY
R. H. A. PLIMMER, D.Sc.
AND
F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

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LECITHIN AND ALLIED SUBSTANCES

THE LIPINS

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PREFACE.

In the present monograph an attempt has been made to present the subject of the lipins in as simple a form as the state of our knowledge permits. The task has been a difficult one, owing chiefly to the chaotic state of the literature dealing with these bodies; the loose manner in which the term "lipoid" has been used has also added not a little to the general confusion. A good deal of space has been given to a description of such alleged lipin substances as protagon and jecorin; it was felt that a detailed discussion of these bodies was necessary in order that the reader might fully appreciate the very insecure and inconclusive evidence on which their existence as chemical entities had been based.

Much of the monograph was written under adverse conditions, and more especially was this the case with Chapter VIII, which deals with the biological aspect of the subject. Before this chapter could be finished I was called to France, and was faced with the alternative of postponing the appearance of the volume indefinitely or of publishing it with this part treated much less fully than I could wish and with the omission of the section dealing with the phenomena of hæmolysis and immunity. The fact that little or nothing is known with certainty of the physiological functions of the lipins suggested that no great loss would ensue from a curtailment of this part, and, after consultation with the Editors, it was resolved to publish the manuscript as it stood. If a second edition should ever be called for, some of the part dealing with the alleged lipins might be curtailed, and a more complete description of the biological aspect of the subject inserted.

Our knowledge of the lipins has been materially increased within the last few years, but, strange as it may seem, we really know little to-day beyond that which was known and published by Thudichum over twenty years ago. Indeed, recent advances are in many cases but corroborations of Thudichum's wonderful experimental work. That the brilliant investigations of this observer should have been so long neglected and despised is an anomaly which perhaps was in some measure due to Thudichum's more or less obscure literary style; his pugnacity towards his opponents may also have contributed to produce this result. Whatever the cause, it is only justice to his memory to affirm that he has done more for lipin chemistry than has been done by any other individual.

H. MACLEAN.

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CHAPTER I.

INTRODUCTION AND NOMENCLATURE.

WHEN animal or vegetable tissues are extracted with ether or with certain other organic solvents, the soluble material consists of ordinary fat together with a variable amount of other substances. Many of these substances are of fatty nature and yield fatty acids on hydrolysis, but differ from neutral fat in that they contain nitrogen or nitrogen and phosphorus in the molecule. Others bear no resemblance whatever to fat except the accidental one of being soluble in the ordinary fat solvents. The ether or alcohol extract of a tissue or organ is therefore composed of a heterogeneous mass of substances which may be roughly classified as follows:—

A. Neutral fat and fatty acids.

B. Substances of varying chemical nature having nor elation to fat such as cholesterol and certain pigments.

The "fat-like" bodies or "Lecithin and allied substances"	C. Substances containing fatty acids, nitrogen and phosphorus. The Phosphatides (Thudichum). Of the phosphatides the best known is lecithin.
	D. Substances containing fatty acids, nitrogen and a carbohydrate group, but no phosphorus. The Cerebrosides (Thudichum).

Many of these "fat-like" bodies are difficult to separate and to obtain in a pure state, and much confusion prevails regarding their number and chemical properties. Various terms have been introduced from time to time to designate these substances, and often they are in a general way referred to as "lipoids". The term lipoid was used by Kletzinski to signify all the unsaponifiable substances that could be extracted from tissues by alcohol and ether. It was reintroduced by Overton [1901], and used by him to denote all those cellular components which dissolve in organic solvents such as chloroform, alcohol and ether. The word "lipoid" is now employed in such a vague and unsatisfactory sense that it is often by no means clear what it is intended to indicate. Some writers use the term with particular reference to the "fat-like" bodies—phosphatides and cerebrosides: others employ it in Overton's sense. Some include neutral fat under the term while others talk of "fats and lipoids". If the word lipoid were used exclusively to denote the "fat-like" bodies its derivation (λίπος = fat) might substantiate this usage. If, as so often happens, it embraces such diverse substances as cholesterol and pigments, the name ceases to have any significance, since the only relationship between these bodies and fat is limited to the physical one of common solubilities in certain solvents.

Any suggestion to restrict the term exclusively to its legitimate groups—the cerebrosides and phosphatides—would simply result in chaos, for in much of the older literature the name would still indicate a more or less extended list of substances according to the particular ideas of the individual author. On the whole it would appear best to drop the word lipoid altogether, for its present usage leads only to confusion and tends to suggest an idea of exactness where none exists.

Under the heading "Lecithin and allied substances" as used in this monograph are included the two classes of "fat-like" bodies mentioned:—

A. The Phosphatides.
B. The Cerebrosides.

These two groups of substances have the property in common of furnishing fatty acids or their derivatives on hydrolysis, and some generic name to include both groups would be of much value. Though in many cases the composition of the bodies comprised by them is still obscure, we have of late years obtained some insight into their general structure, and they are now sufficiently well characterised to justify their inclusion under some designation which would render allusion to them more convenient. For this purpose the term "lipins" is suggested. The word *lipin* was used by Leathes [1910] to denote an indefinite class of substances investigated by Thudichum and called by him amidolipotids. According to Thudichum these bodies (bregenin and krinosin) are compounds of fatty acids, and contain nitrogen, but no phosphorus or carbohydrate group. Their existence in the tissues (brain) as preformed substances is exceedingly doubtful and it seems probable that they are decomposition products. For the present their acceptance as pre-existing substances is unwarranted, and the term "lipin" would seem more suitable as a class name to include the cerebrosides and phosphatides: if future research should substantiate the existence of the amidolipotids they would also be included.

Since the term was employed by Leathes, it has been extensively used especially by American authors, but the sense in which they employ it is entirely different from that suggested by Leathes. Many of these authors use the term virtually to replace the old word "lipoid". Thus Rosenbloom [1911] makes use of the term lipin to designate practically all the known bodies which are extracted from cells by alcohol and ether, along with certain artificial products not found in cells. Under this heading he includes such diverse bodies as lecithin, lipochromes, cholalic acid, lead oleate and cholesterol. The use of the term lipin in this connexion possesses the same disadvantages as the older term lipoid, for there can be no justification for a classification which includes under a common class name such a mixture of chemically unrelated bodies as those mentioned. The terms "lipin" and "lipoid," used in this way, simply indicate the mixture of substances obtained in an alcohol or ether extract of an organ, and it would be preferable in this case for an author to use the terms "alcohol extract," "ether extract" or "alcohol-ether extract" as the case may be. Such a practice, if conveying little information, would at least be exact and logical.

In these pages the term lipin will be employed as a class name to include the cerebrosides and phosphatides. The exclusive use of the word in this sense would simplify matters materially and save much repetition.

Thus in the application suggested the term might be defined as follows:—

Lipins are substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in their molecule either nitrogen, or nitrogen and phosphorus.

CLASSIFICATION OF LIPINS.

Many classifications of these bodies have been attempted, but all are unsatisfactory, owing chiefly to the difficulties experienced in isolating the different substances in a chemically pure form. This difficulty is complicated by the property which many of these bodies possess, of being intersoluble. The result is that mixtures of two or more lipins have from time to time been considered as separate entities and duly given a name. On analysis such mixtures tended to give complicated results, so that an extended and more ample classification was gradually evolved. It will be shown later that the number of lipins, as to whose existence as chemical individuals we have definite evidence, is comparatively small, and it is hoped that the present plethora of unnecessary terms will be gradually eliminated from the literature.

The two groups of bodies (phosphatides and cerebrosides) included under the term lipin have received various names. The term phosphatide, as pointed out by Leathes, has certain disadvantages which are perhaps more theoretical than real, but it does possess the advantage that its application is well defined and indicates a definite group of substances. After all, in dealing with such ill-characterised bodies as the lipins of which it may be said that the exact chemical structure of even one of them is not known with certainty, the nomenclature, seeing it cannot be based on chemical principles, is of minor importance, provided that the sense in which the term is used is strictly defined. The word "phosphatide" fulfils this condition and its long and international usage justifies its retention. The suggestion of Leathes that the term "phospholipines" should be used to replace "phosphatides" has from a certain point of view much to be said in its favour. The present writer hesitates, however, to discard the old term owing to the further confusion which would result in a subject where confusion is already too well marked.

Koch [1903, 8] suggested the name "lecithans" for this class of bodies, but its use has not become general. The cerebrosides have also received different names. No final classification of the lipins can be adopted until research has succeeded in ascertaining the chemical constitution of these substances.

The Phosphatides.

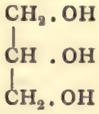
The phosphatides comprise a group of bodies, most of which are of plastic consistency and have distinct fat-like properties. They occur abundantly in eggs, brain, heart, muscle, liver and other organs, and appear to be present in every animal and vegetable cell so far investigated.

Generally speaking, they are soluble in the majority of ordinary solvents for fat, with the exception of acetone in which they are practically insoluble. Advantage is taken of this property to separate them from other fatty substances. They all contain phosphorus, nitrogen and fatty acids, and on hydrolysis yield phosphoric acid (generally in the form of glycerophosphoric acid) together with various fatty acids and basic bodies, such as choline and amino-ethyl alcohol. They form compounds with cadmium chloride, platinum chloride and many metallic salts. They also form combinations with certain organic substances such as protein. Many of them swell up in water to form colloidal solutions from which they can be separated by the addition of sodium chloride and other salts.

Of the phosphatides, the best known are lecithin and the nearly related substance kephalin. A consideration of the structure of lecithin, which may be taken as a type of the phosphatides, at once indicates its relationship to ordinary fat, for we may regard lecithin as a fat in which one of the three fatty acids is substituted by phosphoric acid combined with the base choline.

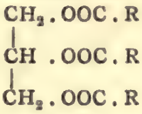
In a neutral fat each of the three (OH) groups of glycerol is replaced by the fatty acid radicle (OOC . R) :—

Glycerol.



$$\begin{array}{l} CH_2 . OH \\ | \\ CH\ . OH \\ | \\ CH_2 . OH \end{array}$$

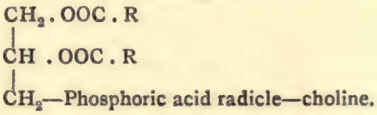
Neutral Fat.



$$\begin{array}{l} CH_2 . OOC . R \\ | \\ CH\ . OOC . R \\ | \\ CH_2 . OOC . R \end{array}$$

and if we consider one of these fatty acid radicles replaced by phosphoric acid, and choline introduced, the resulting product represents the structure of lecithin.

Lecithin.



$$\begin{array}{l} CH_2 . OOC . R \\ | \\ CH\ . OOC . R \\ | \\ CH_2\text{—Phosphoric acid radicle—choline.} \end{array}$$

Kephalin probably possesses a somewhat different structure, but with the exception that the choline of lecithin is represented by another base (amino-ethyl alcohol) the radicles present in both lecithin and kephalin are of the same nature. Besides these two products many other phosphatides are described. There are, however, only two more of whose existence as chemical entities we have any reasonable evidence at present. These are cuorin[1] and sphingomyelin. Cuorin resembles lecithin in general physical and chemical properties, but appears to possess a more complicated structure since Erlandsen [1907] found three fatty acids present in the molecule. The nature of the basic body of cuorin is uncertain, but it is probably amino-ethyl alcohol.

The three phosphatides—lecithin, kephalin and cuorin—all contain fatty acids of the unsaturated series which are exceedingly liable to oxidation changes. On this account these bodies are very labile, and their properties and solubilities soon alter on exposure to light and air, so that their extraction and isolation in an unchanged state is attended with great difficulties. Different varieties of these phosphatides may exist depending on the presence of different fatty acids.

Unlike these bodies, sphingomyelin is a stable substance containing saturated fatty acids, and differs markedly from the other phosphatides mentioned, particularly in its physical properties. It occurs as a glistening white non-hygroscopic powder which does not alter on exposure to air. In many organs only traces of this substance are met with, while in others it has not yet been found. Kidney and brain contain relatively large amounts. Its exact structure is still under discussion.

Besides the above, various phosphatides of doubtful occurrence which have received special names, together with some special bodies which have received no definite names, have been described. These are discussed at a later stage when dealing fully with the individual phosphatides (Chap. VI).

The most general classification of the phosphatides is that introduced by Thudichum in which they are divided into groups depending on the relation of the nitrogen to phosphorus present. Thus, in lecithin and kephalin, one atom of nitrogen and one atom of phosphorus occur so that these bodies represent a N : P ratio of 1 : 1. In the case of cuorin the N : P ratio is 1 : 2, while sphingomyelin has N : P as 2 : 1.

This empirical classification has little to recommend it except expediency, but until our knowledge of these substances is much extended it is difficult to replace it by a better one. All the later classifications on this principle are based on this original sub-division of Thudichum, which can be extended indefinitely to embrace substances with any N : P ratio whatever.

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[1] As will be indicated later (see p. 52) it is not certain that even cuorin represents a unit substance.

The phosphatides described would be classified in the following sub-groups:—

I. Monaminomonophosphatides (N : P = 1 : 1), Lecithin and kephalin.
II. Monaminodiphosphatides (N : P = 1 : 2), Cuorin.
III. Diaminomonophosphatides (N : P = 2 : 1), Sphingomyelin.

The Cerebrosides.

The cerebrosides represent a group of nitrogenous bodies which contain no phosphorus. The members of this group occur chiefly in the brain, and are characterised by the fact that on hydrolysis they furnish the reducing sugar galactose. The cerebrosides are white powders which may be crystalline or of a waxy appearance, thus differing physically from the majority of the phosphatides which are plastic non-crystalline bodies. They tend to occur in nature in company with the phosphatide, sphingomyelin, which, in certain of its solubilities and physical properties, resembles the cerebrosides and is difficult to separate from them. As a result, the literature of the cerebrosides is perhaps more confusing than any other part of the subject of lipins, since a number of different names have been given to bodies which are more or less impure varieties of the same substances, or else mixtures of lipins. At present we know of only two cerebrosides—phrenosin and kerasin.

A Simple Classification of Lipins.

Since the phosphatides and cerebrosides described above are in the writer's opinion the only ones which have been definitely isolated, it is obvious that an elaborate classification is superfluous. The following simple scheme includes them all, and if the future should add to their number, this classification can be easily extended.

Lipins.

I. Phosphatides.	II. Cerebrosides.
(A) *Monaminomonophosphatides* (N : P = 1 : 1) (*a*) Lecithin. (*b*) Kephalin.	(*a*) Phrenosin.
(B) *Diaminomonophosphatides* (N : P = 2 : 1) Sphingomyelin.	(*b*) Kerasin.
(C) *Monaminodiphosphatides* (N : P = 1 : 2) Cuorin.	

Other Classifications.

The change in terminology suggested by Leathes [1910] is undoubtedly the best of the many attempts to place this difficult subject on a better basis, but after careful consideration it was feared that the omission of the older classifications and terminology, and the substitution of Leathes' scheme, was too drastic a step in the present state of our knowledge. Leathes' classification has much to recommend it and possesses the merit of simplicity. All the members of the phosphatides are included under one heading without any attempt at division into groups. On the other hand, though the old sub-grouping of the phosphatides in terms of the N : P ratio is purely empirical, it is in practice not devoid of utility.

Leathes' Classification (1910).

I. PHOSPHOLIPINS (Phosphatides of Thudichum).

Compounds of fatty acids containing nitrogen and phosphorus :—

(*a*) Lecithin.
(*b*) Kephalin.
(*c*) Cuorin.
(*d*) Sphingomyelin.
(*e*) Other related but less completely defined substances.

II. GALACTOLIPINS (Cerebrosides of Thudichum).

Compounds of fatty acids containing nitrogen and galactose but no phosphorus :—

Cerebrone, etc. (phrenosin and kerasin).

For a third group the term lipin is employed to denote components of fatty acids containing nitrogen, but no phosphorus or carbohydrate group.

Other older classifications in which provision is made for a great number of substances, whose existence is doubtful or already disproved, are the following :—

Thudichum's Classification (1884).

I. GROUP OF PHOSPHATIDES :—

Sub-group of Mononitrogenised Monophosphatides (N : P = 1 : 1).

Lecithins.
Kephalins.
Paramyelins.
Myelins.

Sub-group of Dinitrogenised Monophosphatides (N : P = 2 : 1).

Amidomyelins.
Amidokephalins.
Sphingomyelins.

Sub-group of Dinitrogenised Diphosphatide (N : P = 2 : 2).

Assurin.

Sub-group of Nitrogenised Phosphatide Sulphatide.

Cerebrosulphatide.

Sub-group of Non-Nitrogenised Monophosphatides.

Lipophosphoric acid.
Butophosphoric acid.
Kephalophosphoric acid.

II. GROUP OF NITROGENISED NON-PHOSPHORISED PRINCIPLES :—

Sub-group of Cerebrosides.

Phrenosin.
Kerasin.

Sub-group of Cerebrinacides.

Cerebrinic acid.
Sphaerocerebrin.

Sub-group of Cerebrosulphatides.

Body containing sulphur.

Many other substances such as hypoxanthin and amino acids are also included under Group II.

Bang's Classification (1911), (based on Thudichum's).

I. PHOSPHATIDES :—

A. Unsaturated Phosphatides.

(1) *Monaminomonophosphatides* (N : P = 1 : 1).
  (*a*) Lecithin.
  (*b*) Kephalin.
  (*c*) Paramyelin.
  (*d*) Vesalthin.

(2) *Monaminodiphosphatides* (N : P = 1 : 2).
  (*a*) Cuorin.
  (*b*) Liver phosphatide.
  (*c*) Egg yolk phosphatide.

(3) *Triaminodiphosphatides* (N : P = 3 : 2).
  (*a*) Sahidin.
  (*b*) Kidney phosphatide.

B. Saturated Phosphatides.

(1) *Diaminomonophosphatides* (N : P = 2 : 1).
  (*a*) Sphingomyelin.
  (*b*) Aminomyelin.
  (*c*) Apomyelin.
  (*d*) Muscle phosphatide.
  (*e*) Egg yolk phosphatide.
  (*f*) Horse pancreas phosphatide.
(2) *Triaminomonophosphatides* (N : P = 3 : 1).
  (*a*) Neottin.
  (*b*) Carnaubon.
(3) *Protagon.*

C. Phosphatides Insufficiently Characterised.

II. CEREBROSIDES :—
  (1) Phrenosin.
  (2) Kerasin.
  (3) Cerebron.
  (4) Cerebrin and Homocerebrin.
  (5) Pyosin and Pyogenin.

Other classifications have been suggested by Rosenbloom [1911] and by Cramer [1911]. The first of these is based on Leathes' terminology. Cramer suggests the following division of the lipins :—

Cramer's Classification (1911).

(1) PHOSPHATIDES.
Nitrogen-containing fatty acid esters of glycero-phosphoric acid. Some phosphatides may have the glycerin substituted by an unknown alcohol.
  "Lecithin," "Kephalin," "Sphingomyelin," etc.
(2) GALACTOPHOSPHATIDS.
Nitrogen-containing esters of phosphoric and fatty acids with galactose and alcohol groups.
  "Carnaubon."
(3) CEREBROSIDES.
Nitrogen-containing esters of fatty acids and galactose in which no phosphorus is present.
  "Cerebron," "Cerebrin," "Homocerebrin," etc.

(4) PHOSPHOCEREBROSIDES.
Cerebrosides with phosphorus-containing groups.
"Protagon."

These various classifications suffice to show the difficulties connected with the subject of the lipins, and they are given in the hope that the reference to them may be helpful in connexion with substances fully discussed in later pages, where it will be shown that many of the bodies mentioned have no justifiable claims to be included as separate entities in the class of lipins.

CHAPTER II.

THE CHEMISTRY OF THE PHOSPHATIDES: LECITHIN, KEPHALIN, SPHINGOMYELIN AND CUORIN.

ONLY those phosphatides which are sufficiently well characterised to be regarded as chemical entities are described in this chapter. They are lecithin, kephalin, sphingomyelin and with these may perhaps be included cuorin.[1] Of the great number of other substances, which different authors have isolated and regarded as phosphatides, many have received definite names such as vesalthin, neottin, carnaubon and jecorin, while others are referred to in a general way as diaminophosphatides, triaminophosphatides, etc. Many of these bodies are undoubtedly mixtures of lipins; some are lipins mixed with certain nitrogenous impurities, while a few may possibly be true phosphatides which up to the present have not been sufficiently investigated. In this latter class may be included such substances as the myelin of Thudichum. A description and discussion of all these alleged phosphatides is deferred to a later period (Chap. VI) on the ground that their inclusion at this stage would necessarily tend to complicate the subject. Some knowledge of the properties of the better defined phosphatides, their occurrence, extraction and isolation from the tissues (Chap. III) is necessary in order to appreciate the reasons why many of these ill-defined substances have no claim to be regarded as phosphatides. Indeed the existence of any product whatever of the nature of a phosphatide has been denied by Barbieri [1910, 1912], who, in spite of repeated attempts, completely failed to obtain any trace of lecithin from 3000 eggs!

When tissues are extracted with ether or with certain other organic solvents and acetone is added to the extracts thus obtained, the phosphatides are precipitated. Part of this precipitate is soluble in alcohol; this alcohol-soluble fraction, after purification, has a N : P ratio of 1 : 1 and is called lecithin. The alcohol-insoluble part is chiefly kephalin (p. 37) with perhaps a small amount of cuorin (p.

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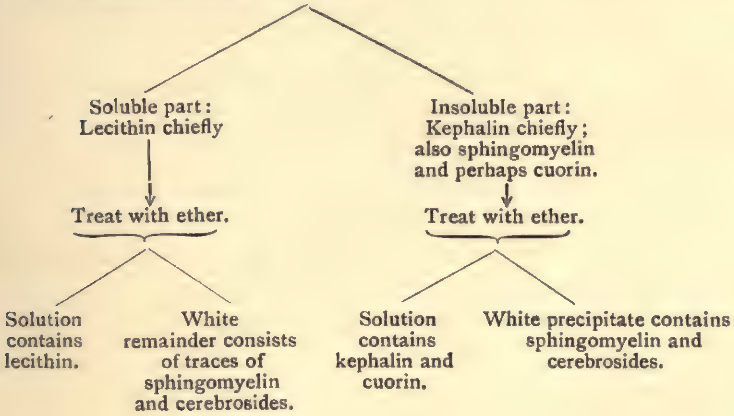
[1] See "cuorin," p. 52.

52). Sphingomyelin (p. 55) may occur in both the alcohol-soluble and alcohol-insoluble portions.

This general relationship is shown in the following scheme:—

Scheme for Rough Separation of Phosphatides.

1. Dried tissue extracted with suitable solvent (ether) and extract filtered.
2. Excess of acetone added to filtrate. Lecithin, kephalin, sphingomyelin, cuorin and cerebrosides are precipitated.
3. *Precipitate extracted with alcohol.*



Soluble part: Lecithin chiefly

Treat with ether.

Solution contains lecithin.

White remainder consists of traces of sphingomyelin and cerebrosides.

Insoluble part: Kephalin chiefly; also sphingomyelin and perhaps cuorin.

Treat with ether.

Solution contains kephalin and cuorin.

White precipitate contains sphingomyelin and cerebrosides.

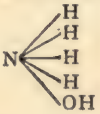
LECITHIN.

The first indication of the occurrence of complex fatty compounds was obtained by Fourcroy [1793]. Later Vauqelin [1812] succeeded in isolating phosphorus-containing fats from the brain. Similar substances, also obtained from brain, were described by Couerbe [1834]. Though these substances were undoubtedly impure, some of them were probably of the nature of "protagon" (p. 120), while others corresponded more or less to lecithin. To one of these bodies isolated by Vauqelin, the name "oleophosphoric acid" was given by Frémy [1841; 1841, 1; 1841, 2]. Frémy and Valencienne [1857] discovered a similar body in the roe of fish. Later Gobley [1846, 1847] isolated a compound of the same nature from egg yolk; this substance [1850] he called lecithin (from λέκιθος, egg yolk). Gobley [1850] first demonstrated the presence of glycerophosphoric acid in lecithin. He also recognised that fatty acids and nitrogen were present, but failed to ascertain the nature of the nitrogenous constituent.

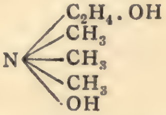
Liebreich [1865] stated that the nitrogen was present as neurine, but this was disproved by Diaconow [1867] and Strecker [1868], both of whom, finding that the platinum chloride compound contained oxygen, came to the conclusion that the base was a body whose platinum chloride combination had the composition $C_5H_{14}NO . Cl . PtCl_2$. Strecker identified it as a body which he had previously [1862] discovered in bile and which he named choline.

Choline possesses the empirical formula $C_5H_{15} . NO_2$ and may be regarded as a substituted ammonium hydroxide in which one hydrogen atom is replaced by the oxyethyl ($C_2H_4 . OH$) group and the other three by methyl (CH_3) groups. Further details are given in Barger's monograph [1914].

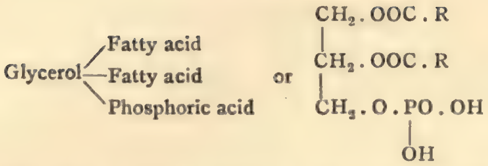
Ammonium hydroxide.



Choline.



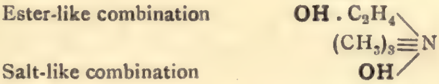
These observations furnished a basis for the chemical constitution of lecithin. Diaconow [1868; 1868, 2; 1868, 3] and Strecker [1868] showed that the fatty acids were combined with the glycerol of the glycero-phosphoric acid in the form of an ester:—



$$\begin{array}{l} CH_2 . OOC . R \\ | \\ CH_2 . OOC . R \\ | \\ CH_2 . O . PO . OH \\ \qquad\qquad | \\ \qquad\qquad OH \end{array}$$

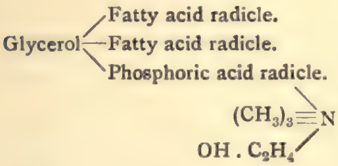
These investigators differed, however, as to the manner in which the choline radicle was attached to the phosphoric acid. Combination could obviously take place by substitution of either of the (OH) groups shown in heavy type in the choline formula.

Choline.

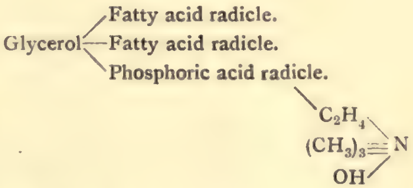


If choline reacted as a substituted ammonium base its combination with phosphoric acid would be through the lower (OH) group to form a salt; on the other hand, choline might act as an alcohol, in which case the combination would be between alcohol and acid constituting an ester. The salt-like combination is represented by formula A in which the (OH) group attached to the nitrogen of the choline is substituted by the phosphoric acid radicle. Formula B represents the ester-like combination in which the (OH) group attached to the carbon (i.e. the (OH) group of an alcohol radicle) is substituted by the phosphoric acid radicle.

Formula A.

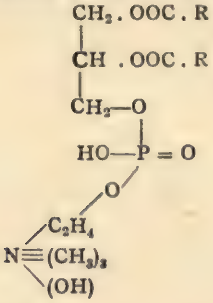


Formula B.



Diaconow [1868, 3] considered lecithin as a compound in which the choline was present in salt-like combination, while Strecker [1868] claimed that the combination was really in the form of an ester. Strecker's observation was confirmed by Hundeshagen [1883] and Gilson [1888], and though recently questioned by Malengreau and Prigent [1912] is still generally accepted. Lecithin is therefore regarded as a body consisting of two fatty acid radicles, one glycerophosphoric acid radicle and one choline radicle, these substances being bound together as represented by the following formula:—

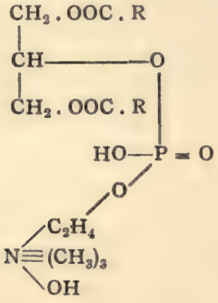
Lecithin Asymmetrical Formula.



This formula contains an asymmetrical carbon atom (the C of the second glycerol group shown in heavy type which is attached to four different radicles or atoms) and the substance should therefore be optically active.

It is obvious from theoretical considerations that it is possible to construct another formula in which the phosphoric acid is linked with the middle carbon of the glycerol, thus

Lecithin Symmetrical Formula.



Such a body would be an isomer of the above, and optically inactive, provided both fatty acids were identical. Since lecithin is optically active [Ulpiani, 1901 ; 1901, 1] and according to Willstätter and Lüdecke [1904] furnishes optically active glycerophosphoric acid on hydrolysis, the asymmetrical formula is generally regarded as the correct one, but the probability of the existence of both isomers must be considered. [Tutin and Hann, 1906; Bailly, 1915; see also "Glycerophosphoric acid," p. 28.]

Various analyses of the percentage of the different elements or units in the molecule have been made by many investigators and the results, though not identical, agree fairly well in many cases. Some of these analyses are given in the table :—

Investigator.	Source of Lecithin.	Percentage Composition.				Molecular Weight.	N : P Ratio.	Equivalent Empirical Formula.
		C.	H.	N.	P.			
Thudichum . .	Brain	66·75	10·67	1·81	4·00	773	1 : 1·00	$C_{43}H_{84}NPO_8$
Erlandsen . .	Heart	65·70	10·21	1·79	3·95	785	1 : 1·00	$C_{43}H_{80}NPO_9$
MacLean . . .	Heart	66·27	10·17	1·87	3·95	—	1 : 1·05	—
Thierfelder and Stern	Egg yolk	64·63	10·96	1·79	3·95	771	1 : 1·00	$C_{42}H_{78}NPO_9$
Baskoff . . .	Liver	64·64	10·71	1·95	4·00	—	1 : 1·08	—

For the estimation of nitrogen in lecithin and other lipins Kjeldahl's method gives good results, while for phosphorus determinations Neumann's [1902, 1904] process is very suitable [Freundler, 1912]. Owing to the presence of phosphoric acid in the molecule, complete combustion of these substances for carbon and hydrogen estimations is attended with some difficulty [Stern and Thierfelder, 1907]. Methods of estimating certain radicles of lecithin are given by Foster [1915]; the estimation of glycerol is described by Erlandsen [1907], Malengreau and Prigent [1912]: of choline by Erlandsen [1907], MacLean [1908]: of the fatty acids by Rollett [1909].

The agreement in the analytical figures is more or less accidental, for it will be shown that the lecithin analysed was in all cases (with the possible exception of Thudichum's sample) a mixture of true lecithin and another lecithin-like substance which has a somewhat similar empirical composition to lecithin.[1]

The constitution of lecithin has been worked out chiefly as the result of investigation of its hydrolytic products. On hydrolysis, lecithin yields glycerophosphoric acid, fatty acids and choline; the glycerophosphoric acid may be partly decomposed giving glycerol and phosphoric acid.

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[1] From various considerations it appears to the author, who hopes to be able to investigate the subject more fully at a later date, that the part of so-called lecithin which has its nitrogen in the form of amino-ethyl alcohol really represents kephalin.

Thus the cadmium chloride combination of this product dissolves in ether just as in the case of the corresponding kephalin combination.

Since lecithin is said to be easily hydrolysed and kephalin is somewhat resistant it might be argued that this of itself indicates that lecithin contains no kephalin. The author, however, has again and again found that certain samples of lecithin were easily hydrolysed to a certain extent, but that a resistant residue remained especially in the case of lecithins with a high percentage of amino-ethyl alcohol.

For the present, therefore, the view is taken that lecithin consists of choline lecithin with amino-ethyl alcohol lecithin, the latter product being really kephalin.

This view is put forward as a purely tentative one, with full recognition of the possibility that it may have to be modified in the future. Obviously the amino-ethyl alcohol lecithin may be of similar constitution to true lecithin, the only difference being in the nature of the base present.

COMPOSITION OF LECITHIN.

A. The Amount of Choline.

The supposed structure of lecithin as indicated above has never been substantiated by quantitative estimation of the products obtained from a given weight of the substance. Many attempts to do so are recorded, especially in the case of the base. Assuming the structure given to be the correct one, it is obvious that the choline represents all the nitrogen of the molecule, so that the amount of choline present could be ascertained from the amount of nitrogen found on analysis. On quantitative estimation of the choline, the amount found should agree with that of the nitrogen present. In practice, however, the figures actually obtained generally fell considerably below the theoretical values. Thus Heffter [1891] using lecithin extracted from liver obtained only 25 per cent. of the theoretical amount of choline, while egg lecithin gave only 77 per cent. [Moruzzi, 1908], heart lecithin 42 per cent. [Erlandsen, 1907], and milk lecithin 39·5 per cent. [Osborne and Wakeman, 1915]. The statement of Coriat [1904] that he obtained theoretical choline values from brain lecithin is contrary to the experience of all other observers.

MacLean carried out many experiments on the subject with lecithins obtained from different sources, and using various modifications in the reagents and method of hydrolysis. In no case was the theoretical yield of choline obtained, though different specimens gave different amounts of the base under similar conditions of experiment. The lecithins were prepared with the greatest care according to the best methods in use at the time, and all had a N : P ratio of approximately 1 : 1. A few samples of commercial lecithin were also used. Some of the results are seen in the table on the opposite page.

It is interesting to observe that commercial lecithin gave the highest percentage of choline; in one sample (Kahlbaum's lecithin) as high a yield as 92 per cent. was obtained [MacLean, 1915]. From a similar specimen of lecithin even higher figures were found by Malengreau and Prigent [1912]. These trade lecithins are prepared from eggs, yet they generally show a much higher choline content than similar lecithins prepared from the same source in the laboratory. For an explanation of this see page 26. In the experiments quoted no effect could be ascribed to the time during which the substance was subjected to hydrolysis. Boiling for two hours or for ten hours gave identical results [Maclean, 1908, 2]. This small yield of the base

Observer.	Source of Lecithin.	Reagent Employed in Hydrolysis.	Time of Boiling in Hours.	Percentage of Nitrogen Obtained as Choline Pt. Chloride. (Averages of several experiments given.)
MacLean [1908]	Commercial specimen "Lecithol" (Riedel)	Baryta in methyl alcohol	2 to 15	76 to 78
,, [1908]	Commercial specimen "Lecithol" (Riedel)	Baryta in water	2½	76 ,, 78
,, [1908, 2]	Ox heart	,, ,, methyl alcohol	2 to 10	37·3 ,, 41·4
,, [1908, 2]	,, ,,	Baryta in water	2 ,, 10	37 ,, 42
,, [1909]	Egg yolk	,, ,, methyl alcohol	3 ,, 6	65 ,, 66
,, [1909]	,, ,,	Hydrochloric acid	1½ ,, 5	64 ,, 65
,, [1915]	Ox heart	Sulphuric acid	30	52

was not due to the destruction of choline by the barium hydroxide or acid used in hydrolysis, for it was shown that only very small traces of choline were destroyed under the conditions of the experiment. [Gulewitsch, 1898; MacLean, 1908, 1]. MacLean [1909, 1909, 1] also showed that when choline was boiled for several hours with substances representing the hydrolytic products of lecithin, it was possible to recover the base in almost theoretical amount. In one experiment choline was boiled for four hours with barium hydroxide solution to which the following ingredients were added:—

Glycerophosphoric acid	1 c.c.
Glycerol	1 c.c.
Phosphoric acid (anhydrous)	0·5 gm.
Stearic acid	0·5 gm.
Oleic acid	0·5 c.c.

The amount of choline added represented 0·2200 gm. choline platinum chloride, and after boiling for four hours and separating the choline in the usual way there was obtained 0·2110 gm. of choline platinum chloride, or 95·5 per cent. of the theoretical amount. More thorough washing of the various residues would no doubt have increased the yield. This experiment proved that the small amount of choline obtained from lecithin was not due to decomposition or to defects in the method for the estimation of choline.

Retention of Nitrogen by Fatty Acid Residues.

Another point observed in these experiments was that the fatty acids or fatty acid soaps obtained on hydrolysis invariably contained a small amount of nitrogen [MacLean, 1908; 1908, 1]. It was quite impossible to obtain nitrogen-free fatty acids however much they were washed. The amount of this residual nitrogen was not large, but it was fairly constant. The figures given below refer to experiments with ox heart lecithin which was hydrolysed by barium hydroxide in methyl alcohol. The barium soaps formed were washed by being rubbed up with ethyl alcohol, boiled for five minutes, allowed to cool and then filtered. This was carried out from one to five times, but even in the latter case the residual nitrogen did not seem to be much reduced. It will be seen from the table that from 5 to 6 per cent. of the nitrogen of lecithin is retained in the fatty acid residue. These results have been corroborated by Trier [1913] and others. Their significance is not at all clear, but the amounts of nitrogen retained are not sufficient to account for more than a small fraction of the loss of choline in lecithin analysis.

No.	No. of Times Washed with 100 c.c. Alcohol: Boiled for Five Minutes each Time.	Mg. of Nitrogen in Lecithin Used.	Mg. of Nitrogen in Residue.	Percentage of Total Nitrogen in Residue.
1	1	8·88	0·490	5·6
2	2	11·50	0·686	5·9
3	3	14·09	0·546	3·9
4	3	9·64	0·504	5·2
5	4	9·01	0·546	6·0
6	5	15·37	0·798	5·2
			Average	5·3

The conclusion drawn from all these experiments was that the whole of the nitrogen of lecithin was not represented by choline as indicated in the generally accepted lecithin formula, but that some other base was also present [MacLean, 1909, 1]. Since different lecithins gave different amounts of choline, and even lecithins obtained from the same source but isolated by different methods (egg lecithin of commerce and egg lecithin obtained in the laboratory) gave varying amounts of this base, it was most reasonable to assume that lecithin was really a mixture of two substances of somewhat similar composition, one of them having choline as its base and the other some unknown nitrogenous body. The recent suggestion of Darrah and MacArthur [1916] that lecithin is a *combination* containing 1 molecule of choline and 1 molecule of another base (amino-ethyl alcohol) is entirely contradicted by the fact that such variable amounts of choline are found in different lecithins.

These suggestions, indicating that lecithin contained another base besides choline, were confirmed by the discovery of Trier [1911] who found β-amino-ethyl alcohol, $CH_2(NH_2)CH_2 . OH$ [see Barger, 1914], among the products of hydrolysis of the phosphatides of bean meal (*Phaseolus vulgaris*). Later, the same observer recovered amino-ethyl alcohol from egg lecithin, identifying it by means of the gold chloride combination [1912, 1; 1913]. Amino-ethyl alcohol was also discovered independently in egg lecithin by Eppler [1913] working in Thierfelder's laboratory and has been found in several specimens of heart lecithin [MacLean, 1915]. It has also been obtained recently from various other lecithins both of animal and plant origin. Amino-ethyl alcohol is very difficult to estimate in the ordinary way, since all its combinations are exceedingly soluble, but it can be very easily estimated indirectly by Van Slyke's method for determining amino nitrogen [1912] since all its nitrogen is readily given off on treatment with nitrous acid. All the amino nitrogen present in lecithin is probably present as amino-ethyl alcohol, since there is now abundant evidence that this base is always present, and no other base containing amino nitrogen can be obtained from lecithin. Thierfelder and Schulze [1915], however, suggest that a small amount of some other base is also present.[1] From alcoholic solutions of hydrolysed lecithin, platinum chloride precipitates only choline; a combination of amino-ethyl alcohol platinum chloride remains in solution. It is on account of the extreme solubility of the compounds of this base that it had been missed for so long. The presence of two bases in lecithin being established, it was now of interest to determine whether the whole of the nitrogen of lecithin could be accounted for by the choline and amino nitrogen present.[1]

Distribution of Lecithin Nitrogen as Choline and Amino-Ethyl Alcohol.

To determine whether lecithin might possibly contain some base other than the two mentioned, MacLean [1915] carried out experiments with hydrolysed and ordinary unhydrolysed lecithin. The choline was determined in the usual way as the platinum chloride compound, while the amino nitrogen was estimated by Van Slyke's method, using in the majority of cases his micro form of apparatus [1913].[1]

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[1] Since the quantitative estimation of amino-ethyl alcohol has been carried out by various observers indirectly by estimating the amino nitrogen, it is obvious that the results obtained do not exclude the possibility of some other amino substance being present.

(*a*) *Experiments with Unhydrolysed Lecithin.*

Attempts to estimate the amino nitrogen in unhydrolysed lecithin were rendered difficult, partly on account of the frothing which resulted, and partly owing to the lack of a suitable solvent for lecithin. The solvent used was strong acetic acid, but it was found that after the preliminary shaking of the sodium nitrite and acetic acid, according to the directions of Van Slyke, the addition of acetic acid (instead of the usual aqueous solvent or very weak acid or alkali) acted in such a way as to give a good deal of gas which was not taken up by the permanganate absorbing mixture. This introduced an error which had to be allowed for by controls, but even then the results were not satisfactory. In spite of these disadvantages very suggestive numbers were obtained. The table shows the results of five experiments. The choline was estimated by hydrolysis of the lecithin with weak acid followed by the ordinary procedure.

No.	Amino Nitrogen Found in Percentage of Total Nitrogen.	Choline Nitrogen Found in Percentage of Total Nitrogen.	Nitrogen Retained by Fatty Acids in Percentage of Total Nitrogen.	Percentage of Total Nitrogen Accounted For.
1	31·4	66	4	101·4
2	30	68·6	3	101·6
3	38·2	63	4·4	105·6
4	28	64	6	98
5	30	68	6	104

These results, though not conclusive, suggested that all the nitrogen of lecithin, not represented by choline, was present in the amino form—as amino-ethyl alcohol. This is better brought out in the next set of experiments, where hydrolysed lecithin was used for the amino nitrogen estimation and no experimental difficulties were encountered.

(*b*) *Experiments with Hydrolysed Lecithin.*

In these experiments hydrolysis was carried out with weak sulphuric or hydrochloric acid. For technical considerations it was found more convenient in some cases to estimate the choline in one portion of the lecithin, a separate portion of the same sample being used for the amino-ethyl alcohol determinations. This was done in two experiments. In other cases aliquot parts of the same filtrate were taken. The amounts of nitrogen given are in terms of the total nitrogen in the filtrates obtained after hydrolysis.

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[1] See Plimmer's monograph, Part I, Analysis, 1917.

Experiment.	Source of Lecithin.	Total Nitrogen in Filtrate.	Percentage of Nitrogen as Choline.	Percentage of Nitrogen as Amino-Ethyl Alcohol.	Percentage of Total Nitrogen of Filtrate.
1	Egg yolk	17·5	74·6	25·7	100·3
2	,, ,,	16·5	78·8	23·6	102·4
3	Ox heart	20·2	74·2	24·5	98·7
4	Horse kidney	23	66·5	34·8	101·3
5	Egg yolk	—	66·4	35·8	102·2
6	,, ,,	—	70	31	101

From these results there can be no doubt that the whole of the water-soluble nitrogen of lecithin is present in the form of choline and amino-ethyl alcohol. It is probable that the nitrogen retained in the fatty acids was also in the amino form, though some of this might be due to traces of impurity in the lecithin; it is worth noting that in many cases the nitrogen content of the lecithin is often slightly higher than is required to give a N : P ratio of 1 : 1. At any rate, the part retained by the fatty acids is small.

When an alcoholic solution of choline containing amino-ethyl alcohol is precipitated by platinum chloride it frequently happens, if the solutions are concentrated, that some amino-ethyl alcohol platinum chloride is carried down with the choline double salt. This was found to be the case to a very slight extent in the above experiments. Here the amino nitrogen actually present in the platinum chloride precipitate was estimated and due allowance made. In this connexion, it is best not to have the alcoholic choline solution too concentrated; again the precipitate should be very thoroughly washed with absolute alcohol before drying. It must be dried at 105° to remove water of crystallisation. In pure alcoholic solution, synthetic amino-ethyl alcohol gives a distinct precipitate with platinum chloride, especially if the latter is added in excess, but in the presence of glycerol and other impurities the double salt tends to remain in solution.

Separation of Lecithin into its Components.

So far it was not known definitely whether lecithin was a mixture of two phosphatides or a complicated compound containing two bases. Indeed it was not proved that any body corresponding to the accepted lecithin formula really existed. MacLean [1915], however, succeeded in separating lecithin into its components and obtained two substances, one of which had all its nitrogen in the form of choline, and presumably possessed the formula ascribed to lecithin. The other substance present was not obtained in quite pure form, but it was almost certainly kephalin.

It will thus be seen that the term "lecithin" as generally employed refers to a body which is soluble in alcohol, and which represents a mixture of true lecithin[1] and kephalin in varying proportions. The amount of kephalin present depends largely on the methods adopted in the preparation of the lecithin, but it may be said in a general way that the greater the precautions taken the more kephalin does the lecithin contain (Chap. III).

The method adopted for the separation of these two bodies depends on the different solubilities of the cadmium chloride compounds of lecithin and kephalin. MacLean [1909, 1] showed that when an alcoholic solution of lecithin is treated with cadmium chloride, the percentage of nitrogen present as choline in the lecithin precipitated as the cadmium chloride compound, is greater than that of the original lecithin; on the other hand, the substance remaining in the filtrate has correspondingly less of its nitrogen represented by choline.

This observation, which was confirmed by Trier [1912] and by Eppler [1913], suggested a possible method for the fractionation of lecithin into its components, but it was not found possible to effect complete separation by this means. By the use of ether, however, isolation of a true lecithin, containing only choline, was accomplished. The original lecithin was dissolved in alcohol, and excess of cadmium chloride added. The resulting precipitate was thoroughly digested with ether which dissolved the kephalin part, leaving true lecithin cadmium chloride behind as a white residue. This ether-insoluble compound was dissolved in a mixture of ethyl acetate and alcohol, from which it separated on cooling in the form of beautiful white clusters of well-formed needles arranged in stars and rosettes. In one case the ether-insoluble cadmium chloride compound was divided into two parts—a benzene-soluble and a benzene-insoluble part—before treatment with the mixture of alcohol and ethyl acetate. Analysis showed that practically the whole of the nitrogen of the ether-insoluble cadmium chloride combination was present as choline. In the experiment quoted the numbers refer to the total nitrogen of the filtrate after hydrolysis; no account is taken of the small amount of nitrogen retained by the fatty acids or other residue. The lecithin used was obtained from ox heart. The amino nitrogen was estimated in the filtrate after the separation of choline as the platinum salt.

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[1] The term "lecithin" is used in this monograph in the sense in which it is usually employed—i.e. to designate what is now known to be a mixture of lecithin and kephalin. The part of this mixture represented by the phosphatide which has all its nitrogen in the form of choline and agrees with the theoretical formula for lecithin is referred to as "True Lecithin".

No.	Substance.	Percentage Nitrogen as Choline.	Percentage Nitrogen in Amino Form.	Percentage of Total Filtrate Nitrogen Accounted for.
1	Lecithin	62	37·2	99·2
2	Cadmium chloride pp. from (1)	65	33	98
3	Ether insoluble fraction of (2) (benzene sol.)	94	3	97
4	,, ,, ,, ,, (2) (,, insol.)	99	None	99
5	,, soluble ,, ,, (2)	45	54	99

Here the two fractions (3) and (4) contained all their nitrogen (with the exception of a trace in No. 3) as choline. A cadmium chloride compound of a true lecithin, having all its nitrogen as choline, had therefore been separated from an original lecithin which had only 62 per cent. of its nitrogen as choline.

On separation of the free phosphatide by Bergell's method [1900] a sample of true lecithin was isolated which had all the properties of ordinary lecithin, and from which the theoretical amount of choline was obtained.

Though it was not possible to get the ether-soluble cadmium chloride fraction quite free from true lecithin, there seems to be little doubt that the other substance present was kephalin,[1] which contains the whole of its nitrogen in the amino form.

From the results of these experiments, lecithin must be regarded as a mixture of true lecithin and kephalin, so that the figures obtained in lecithin analyses, though often in agreement with each other, are worthless as an indication of purity. The subject is discussed later in considering the methods employed for the preparation and isolation of lecithin (Chap. III). In this connexion it is interesting to note that a lecithin prepared from the cadmium chloride compound which had been washed with ether was found by Lüdecke [1905] to contain 96 per cent. of the theoretical amount of choline. It is probable that some of the commercial lecithins which show a high percentage of choline are prepared from the washed cadmium chloride combination by Bergell's method [1900]. This would account for their comparatively high choline content.

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[1] See p. 17.

B. The Other Hydrolytic Products of Lecithin.

(*a*) *Fatty Acids of Lecithin.*

Our knowledge regarding the nature of the fatty acids of lecithin is very incomplete. The lecithin formula admits only of the presence of two fatty acid radicles in the molecule, but various observers have obtained three or even four different fatty acids among the hydrolytic products of lecithin, while others could find only one. Thus Diaconow [1868] stated that the only fatty acid present was stearic ($C_{18}H_{36}O_2$), while Strecker [1868] obtained palmitic acid ($C_{16}H_{32}O_2$) and oleic acid ($C_{18}H_{32}O_2$), but no stearic acid. Diaconow [1868, 3] attempted to explain these differences by assuming the existence of various lecithins each containing different fatty acids. As proof of this assertion he stated that a sample of lecithin which was obtained in the form of a precipitate by cooling an alcoholic extract of egg yolk gave only stearic acid. On the other hand, the filtrate from the cooled alcohol extract contained a lecithin which furnished both stearic and oleic acids. The first of these lecithins corresponded to distearyl lecithin, while the second one would be regarded as stearyl-oleic lecithin. By a similar process of reasoning Strecker's palmitic acid was accounted for on the ground that the method of isolation adopted by the latter observer yielded a lecithin containing palmitic acid. Since observations on the iodine value of lecithin suggest that all lecithins contain at least one unsaturated acid, the existence of such compounds as distearyl lecithin or dipalmityl lecithin is highly improbable. It is obvious, however, that the whole question of the fatty acids of lecithin is complicated by the fact that lecithin is a mixture of true lecithin with a greater or less amount of kephalin. In any case, some of the fatty acids must have come from kephalin. Thudichum [1884], who worked with a specimen of lecithin which, from its method of preparation, must have contained little or no kephalin, obtained oleic acid and an acid having the composition of the so-called margaric acid

($C_{17}H_{34}O_2$).[1] Stearic and oleic acids were found by Ulpiani [1901, 1], while Cousin [1903], using egg-yolk lecithin, obtained stearic, palmitic, oleic and linolic acids; from brain lecithin he isolated similar products, though he was unable to identify linolic acid with certainty [1906].

Other results include stearic, palmitic and oleic acids from brain lecithin [Koch, 1902] and from lecithin from ovaries and testicles of tunny fish [Dezani, 1909]. Palmitic and oleic acids from egg-yolk lecithin [Serano and Palozzi, 1911] and oleic, linolic and stearic acids from commercial lecithin [Fourneau and Piettre, 1912].

(*b*) *Observations on the Fatty Acids obtained from Reduced Lecithin.*

Since part at least of the fatty acids obtained from lecithin is unsaturated, and is consequently very liable to undergo oxidation changes during the various processes necessary for its isolation, it seems probable that some of the above investigators were dealing with mixtures of partially oxidised acids. The difficulty of preventing oxidation appears to be very great, and to this may be ascribed, to some extent at least, the difficulties encountered in ascertaining the nature of the fatty acids in this product. To avoid these oxidation changes Rollett [1909] recommended the use of tin or zinc, which in the presence of acid formed hydrogen and thus prevented access of oxygen.

Various attempts have been made to reduce lecithin by saturating it with hydrogen in the presence of a suitable catalyst, and so to prevent as far as possible oxidation of the unsaturated acids. Paal and Oehme [1913] reduced egg lecithin by saturating a solution of this body in 90 per cent. alcohol with hydrogen in the presence of colloidal palladium. The resulting product was found to be a crystalline substance which was much less soluble in alcohol than was ordinary lecithin, and which could be recrystallised from hot alcohol. On hydrolysis this saturated lecithin furnished much stearic acid, a result which probably indicated that the original unsaturated acids present belonged to the C_{18} series. Palmitic acid, however, was also found, and these observers inclined to the view that the original lecithin contained palmitic and linolic acids.

Ritter [1914] could obtain only stearic acid from lecithin reduced by the method used by Paal and Oehme. He concluded, therefore, that the acids of lecithin belonged entirely to the C_{18} series [see also Riedel, 1913, 1914].

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[1] It is now recognised that the substance formally called margaric acid was not a chemical entity but probably a mixture of palmitic and stearic acids.

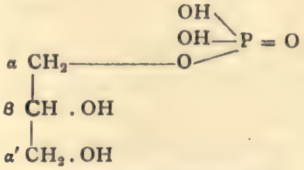
All these divergent results might be explained by Diaconow's suggestion that different lecithins exist characterised by the presence of different fatty acids in the molecule, and this possibility must not be overlooked. It seems more probable that the true explanation lies in the fact that some of these acids had come from the kephalin present. Lecithin, as ordinarily prepared, would be expected to furnish four acids, and since kephalin is responsible for two of these, the remaining two must belong to the true lecithin part. The kephalin acids are stearic and linolic acids (p. 39), and since the only other acids obtained from lecithin are apparently palmitic and oleic acids, it follows that the fatty acids of true lecithin should be palmitic and oleic acids. This, however, is only conjecture, and at present no definite statement can be made, but the available evidence suggests that one at least of the acids of lecithin is of the unsaturated variety and is probably oleic acid; the other acid appears to be saturated and is either palmitic or stearic acid. These difficulties should be easily settled by examining a sample of true lecithin, an investigation which recent results have now rendered possible.

(*c*) *Other Bases of Lecithin.*

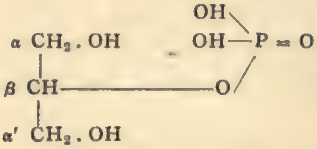
Besides amino-ethyl alcohol and choline already described, several investigators have suggested that other bases might be present. Thus Berlin [1912] states that homo-choline might probably substitute choline in certain lecithins. So far no definite evidence of the presence of any other basic body has been produced, and it is probable that the indications obtained by Thierfelder and Schulze [1915] that another base exists in lecithin depended on the presence of impurities in the lecithin employed.

(*d*) *Glycerophosphoric Acid of Lecithin.*

From the chemical standpoint this acid can exist in two modifications—the *α*- and *β*-forms—and is really the glycerine ester of phosphoric acid.



α-form (unsymmetrical).



β-form (symmetrical).

The *a*-form contains an asymmetric carbon atom, and is therefore capable of existing in optically active forms whilst the *β*-variety is inactive. After the discovery of Willstätter [Willstätter and Lüdecke, 1904; Lüdecke, 1905; see also Mayer, 1906] that the glycerophosphoric acid of egg lecithin was optically active, rotating the plane of polarised light to the left, it was generally assumed that the glycerophosphoric acid of lecithin was the *a*-form. This observation of Willstätter, while proving the presence of *a*-glycerophosphoric acid in lecithin, did not exclude the possibility of some of the *β*-variety being present as well, and Tutin and Hann [1906] maintained that lecithin contained a mixture of *a*- and *β*-glycerophosphoric acids. The question was by no means easy to settle owing to the difficulties of identifying the two forms, but recently Grimbert and Bailly [1915, 1915, 1] introduced a simple chemical test, by means of which it is possible to determine whether these monoglycerophosphoric esters are of the *a*- or *β*-varieties. These observers pointed out that an *a*-ester, on careful oxidation so as to avoid hydrolysis, should yield a compound containing a ketone group which could be identified by Denigès' reaction [1909]. On the other hand, the *β*-form would not give this reaction. The oxidising agent used was a ·25 per cent. solution of bromine in water; to prevent any hydrolysis by the nascent hydrogen bromide, the reaction was carried out in the cold. By means of this test Bailly [1915] claimed to have definitely shown that egg lecithin is a mixture of at least two isomers containing the symmetrical and asymmetrical varieties of glycerophosphoric acid. Indeed, he states that the inactive symmetrical form predominates.

It is probable, therefore, that lecithin contains a mixture of the *a*- and *β*-forms of glycerophosphoric acid; in many cases the product obtained on hydrolysis is optically inactive, and consists to some extent of the racemic *a*-form, but whether any of the dextro-rotatory form actually occurs as such in lecithin is unknown. Probably it does not.

According to Malengreau and Prigent [1912] lecithin glycerophosphoric acid is fairly resistant to acids, and is more readily hydrolysed by acids in weak concentration than by stronger solutions. It is readily decomposed by a ferment present in the intestines, kidneys and other tissues [Grosser and Husler, 1912].

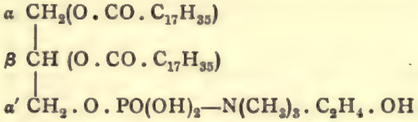
Though the literature on the naturally occurring glycerophosphoric acid is comparatively small, much work has been done on the synthetic product. For the chief results obtained in this connexion, in addition to references already given, the following may be mentioned: Adrian and Trillat [1898], Carré [1904, 1912, 1912, 1], Power and Tutin [1905], Malengreau and Prigent [1911], Neuberg and Tir [1911], Neuberg and Kretschner [1911], Contardi [1912], Rogier and Fiore [1913], King and Pyman [1914], Bailly [1915, 1].

So far our knowledge of lecithin is after all very meagre. We know that it is a mixture of two substances with different bases, but whether the α-glycerophosphoric acid is the acid of one base and the β-form the acid of the other one, or whether both bases are attached to both varieties of acid we do not know; obviously the number of possible combinations is great. Further, the nature of the fatty acids present, and the question whether various homologues of lecithin containing different fatty acids exist, are problems still shrouded in obscurity.

ATTEMPTS AT SYNTHESIS OF LECITHIN.

For such a complicated product as lecithin it is obvious that a more complete knowledge of its constitution than we at present possess must precede all successful endeavours to synthesise it. The old idea that lecithin represented a choline ester of a distearylphosphoric acid formed the basis, in a few cases, for attempts at synthesis of this product. Up to the present, the results have not been satisfactory, though certain claims have been advanced.

The first important work in this connexion was carried out by Hundeshagen [1883], who by treating distearylphosphoric acid with choline carbonate obtained a choline-distearyl-glycero-phosphate. Here the choline reacted as a substituted ammonium base, so that the product was a choline salt and not an ester compound as in the case of lecithin.



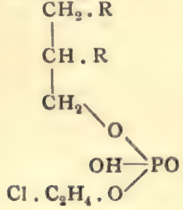
$$\begin{array}{l} \alpha\ CH_2(O.CO.C_{17}H_{35}) \\ \quad | \\ \beta\ CH\ (O.CO.C_{17}H_{35}) \\ \quad | \\ \alpha'\ CH_2.O.PO(OH)_2{-}N(CH_3)_3.C_2H_4.OH \end{array}$$

Hundeshagen prepared his distearin by Berthelot's method [1854], and by heating this with phosphoric anhydride he obtained distearylglycerophosphoric acid. Since Berthelot's method gives chiefly *a*, *a'*-distearin [Grün, 1905], it is probable that Hundeshagen's product was largely or altogether composed of the choline derivative of this compound. All attempts to combine the choline in ester form failed.

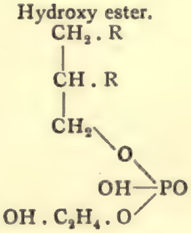
Gilson [1888] endeavoured to overcome this difficulty by treating distearylphosphoric acid with ethylene oxide and trimethylamine, but here also the result was unsatisfactory.

The problem was recently taken up by Grün and Kade [1912, 1912, 1], who treated distearin with phosphorus pentoxide and glycol chlorhydrin. The resulting product consisted of a mixture of two esters—an hydroxyethyl ester and a chlorethyl ester—formed respectively by the interaction of the $CH_2.Cl$ and the $CH_2.OH$ groups of chlorhydrin with the phosphoric acid :—

Chlorethyl ester.

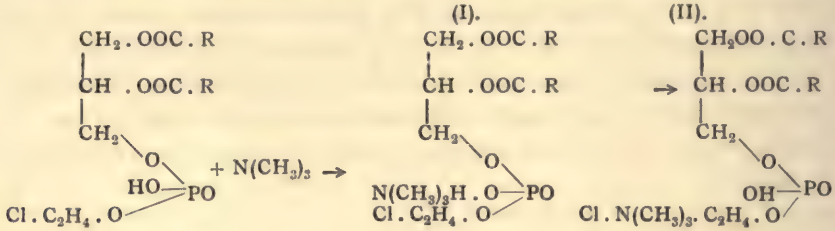


Hydroxy ester.



By heating the product with thionyl chloride the hydroxyethyl ester was converted into the chlorethyl ester so that a single substance resulted.

This chlorethyl ester was then treated with trimethylamine, the first stage in the reaction being the formation of the trimethylammonium salt of chlorethylphosphoric acid (I), which in the presence of excess of trimethylamine was converted into the choline chloride ester of distearylphosphoric acid (II) (lecithin chloride).



About 1·5 gms. of this compound were obtained, but though the analysis, as far as it went, appeared to agree fairly well with that required by the theory, the authors do not seem to be quite satisfied that the compound obtained was actually pure lecithin chloride as represented by the above formula. It is interesting to observe that this product agreed with lecithin in its general solubility in many organic solvents.

Experiments to convert the chlorethyl ester of phosphoric acid into the choline ester by the action of trimethylamine were first carried out by Langheld [1911]. He studied the reaction of ethyl metaphosphate with chlorhydrin, and obtained the ethyl chlorethyl ester of orthophosphoric acid. This substance, on treatment with trimethylamine, gave choline ethyl phosphate, the choline being attached in ester form. No attempts to combine this with distearin in order to form a product similar to lecithin were carried out.

CHIEF PROPERTIES OF LECITHIN.

Freshly prepared lecithin is a yellowish-white waxy substance, which on exposure to air absorbs oxygen and soon assumes a dark brown colour. It is very hygroscopic, and in the presence of moisture forms a soft plastic mass. On thoroughly drying *in vacuo*, it becomes hard and somewhat brittle so that it can be ground to a coarse powder.

Like various other lipins, lecithin forms "liquid crystals" [Lehmann, 1914; see also p. 101]; the so-called myelin forms which appear on warming an aqueous suspension of lecithin are closely connected with this phenomenon.

Lecithin dissolves very easily in alcohol, ether, chloroform, benzene, petroleum ether and many other organic reagents; also in aqueous solutions of bile salts [Long and Gephart, 1908, 1]. With some of these solvents it forms true solutions, while in the case of others the solutions are probably colloidal [Loewe, 1912, 3]. In acetone and methyl acetate it is insoluble. In contact with water it swells up and ultimately, if sufficient fluid be present, forms a slimy emulsion or colloidal solution from which it can be readily precipitated by salts with divalent cations, such as calcium and magnesium [Koch, 1903, 1907, 1909; Porges and Neubauer, 1907]; salts containing monovalent cations (NaCl, etc.) act in the same way, but more slowly [Long and Gephart, 1908]. In the presence of a small amount of sodium chloride, acetone readily precipitates lecithin from its emulsions with water [MacLean, 1912, 1912, 3]. It is also readily precipitated from ether or chloroform solution by this reagent; from alcoholic solution acetone precipitates it in many cases rather slowly and incompletely, while, from ether solution, precipitation is complete in the presence of a small amount of magnesium chloride [Nerking, 1910]. All the precipitations are apparently hastened by the presence of traces of neutral salts. Though insoluble in pure acetone, lecithin may dissolve to some extent in acetone containing fat or fatty acids, so that its separation from other fatty bodies by means of acetone is always incomplete.

The so-called melting-point of lecithin is indefinite, but is somewhere in the region of 60°; at 110° it decomposes and forms a brown mass.

On treatment with alkalies or acids [Moruzzi, 1908; MacLean, 1908] it is quickly hydrolysed on heating, and more slowly in the cold; it is slowly decomposed by picric acid at room temperature [Lüdecke, 1905]. Various enzymes also decompose it [Bergell, 1901].

Electrolytes decrease both the osmotic pressure and viscosity of aqueous lecithin suspensions, while lipin solvents, on the other hand, increase both [Handovsky and Wagner, 1911; Berezeller, 1914; Thomas, 1915].

Investigation of the distribution of dyes (methylene blue) between an aqueous phase and a chloroform phase, in which lecithin and various other lipins were dissolved, indicated that the distribution of the dye did not obey Henry's law, but appeared to follow the formula introduced by Freundlich for adsorption phenomena [Loewe, 1912].

Lecithin owes many of its chief properties to its colloidal nature. When shaken with chloroform, a lecithin emulsion becomes opalescent due to adsorption of the chloroform [Calugareanu, 1910]. When added to a solution of albumoses part of the albumose is precipitated in the form of an adsorption compound. This and other reactions of lecithin with proteins and enzymes occur in exactly the same way when lecithin is replaced by mastic, showing that the phenomena are all directly dependent on the colloid nature of lecithin [Michaelis and Pincussohn, 1906; Michaelis and Rona, 1907].

Lecithin combines with acids and bases. Bing [1901] describes a sodium-chloride-lecithin, soluble in ether but insoluble in alcohol, and precipitated from ethereal solution by alcohol. Addition compounds of lecithin with certain salts of the heavy metals, such as cadmium chloride, platinum chloride and mercuric chloride are well known. Lecithin-cadmium-chloride is almost insoluble in alcohol, but dissolves in a mixture of carbon disulphide and ether or alcohol. Analyses of this combination by various observers have yielded different figures for the cadmium content, but this was obviously dependent on the nature of the lecithin used, on the amount of washing with ether or other solvents, and on other factors. Indeed, such divergent results were just what might have been expected in the case of a variable mixture like lecithin. Both platinum chloride and mercuric chloride salts are insoluble in alcohol but soluble in ether. A molybdenum compound is also described [Ehrenfeld, 1908]. Lecithin is not precipitated from its alcoholic solution by lead acetate and ammonia [Thudichum, 1884].

Combinations of protein with lecithin such as vitellin and lecithalbumin are recognised, though our knowledge of these substances is as yet incomplete [Liebermann, 1893; Christen, 1905; Galeotti and Giampalmo, 1908; Mackenzie-Wallis and Schölberg, 1910; Handovsky and Wagner, 1911; Feinschmidt, 1912].

It is probable that the greater part of the lecithin of tissues exists in some kind of combination with protein. Inorganic material has also been found in lecithin, but it is improbable that this was present in true combination. Such inorganic substances are iron [Glikin, 1908; Burow, 1910; Altschul, 1912], calcium and chlorine [Stern and Thierfelder, 1907].

Compounds of lecithin with salts of organic acids such as sodium lactate and potassium oxalate are also known.

Several products containing sugar and lecithin occur in nature or have been prepared artificially (see Jecorin, p. 152), but it is probable that these are adsorption complexes; no definite combination between lecithin and carbohydrate in the chemical sense has been established, though Scott [1916] obtained indications that such combinations may possibly exist. According to Porges and Neubauer [1907] the power of lecithin to make ether-insoluble substances, such as sugar, soluble in ether is due to the fact that it is a hydrophile colloid, and so combines with water and in this way takes up certain water-soluble substances.

Lecithin does not generally act as a true solvent, but rather as an adsorbent [Loewe, 1912, 2]. Many protein colloids which dissolve only in water are readily soluble in organic solutions, such as chloroform when lecithin is present [Michaelis and Rona, 1907].

Combinations of lecithin with glucosides and alkaloids [Bing, 1901], snake venom [Kyes, 1903], cholesterol and pigments [Overton, 1900], and enzymes [Michaelis and Rona, 1907] have been described, but all of them are insufficiently studied.

In many cases it is difficult to say whether we are dealing with true compounds of lecithin, adsorption complexes or merely mechanical mixtures. Probably the majority of the so-called combinations are adsorption compounds [Loewe, 1912, 1].

Lecithin is very labile, and undergoes changes as the result of oxidation, gradually becoming insoluble in alcohol and in ether and tending to become soluble in water. A highly oxidised specimen of lecithin may therefore differ materially from a fresh specimen in solubility and other properties, and no doubt this phenomenon accounts for certain alleged new varieties of lecithin which have been described.

This tendency to oxidation makes it necessary to adopt certain precautions when working with lecithin. Air and light should be excluded as far as possible, and the reagents employed should be quite pure [Erlandsen, 1907; Heubner, 1908; Stern and Thierfelder, 1907, etc.]. Lecithin should be preserved *in vacuo* in a dark desiccator and all operations completed in the minimum time. This liability of lecithin to oxidation is dependent on the presence of unsaturated fatty acids in the molecule, and is much increased by the presence of various chemical reagents, such as iron salts [Thunberg, 1909, 1913; Warburg, 1913; Warburg and Meyerhof, 1913], potassium dichromate [Thunberg, 1916] and magnesium and manganese compounds [Ciaccio, 1915]. The absorption of oxygen by the lipins of the spinal cord has been demonstrated by means of the micro-spirometer [Signorelli, 1910]. According to Long [1908] an emulsion of lecithin in aqueous solution is fairly stable, but this statement must be accepted with reserve.

Lecithin absorbs iodine and bromine,[1] and saturated halogen derivatives of lecithin have been described [Riedel, 1905]. The "iodine value," which serves as a good indication of the degree of desaturation of the fatty acids present, also gives useful information as to the extent to which oxidation has taken place. The lowering of the iodine value consequent on oxidation accounts largely for the different results obtained by various observers. Thus for egg lecithin Rollett [1909] found a value of 69, while Stern and Thierfelder [1907] obtained only 48·7 for their specimens. Among other figures recorded are 100 for heart lecithin (Erlandsen), 63 for liver lecithin (Baskoff), while Cruickshank [1914] obtained for lecithin from ox heart 72, from liver 81, from testicles 117, and from thyroid 69.

Lecithin prepared at the ordinary temperature as well as the corresponding cadmium chloride compounds, is dextro-rotatory. By heating an alcoholic lecithin solution at 90 to 100° in a closed tube for some time, the optically active body becomes inactive. According to Mayer [1906, 1908] *l*-lecithin may be obtained from this inactive racemic preparation by treating it with lipase, when the *d*-lecithin is decomposed leaving the *l*-variety untouched. The accuracy of this observation, however, has been contested by Bang [1907].

A number of colour reactions depending on the presence of unsaturated fatty acids have been described [Thudichum, 1884; Struve, 1900; Casanova, 1911].

[1] See "Actien Gesellschaft für Anilin Fabrikation" [1905].

KEPHALIN.

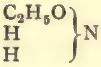
Kephalin is a phosphatide in many respects closely resembling lecithin, with which it seems to be associated in all tissues [Koch and Woods, 1905]. It is distinguished from lecithin by its insolubility in alcohol; the separation of lecithin and kephalin from a mixture of both lipins is based on this property.

Kephalin was first investigated by Thudichum [1884], who isolated it from a brain extract and suggested its probable composition. There is no doubt that Thudichum's sample was impure, but although various improvements in the methods of isolating and purifying kephalin have since been introduced, it is still very doubtful whether a chemically pure specimen has been obtained.

As the result of hydrolysis of kephalin, Thudichum found that it contained two fatty acids, (1) an acid identical with, or resembling, stearic acid which differed in different specimens of kephalin, and (2) a special acid which he considered peculiar to kephalin and called kephalic acid. He also isolated glycerophosphoric acid and three basic bodies:—

(1) Choline (which he always refers to as "neurin").[1]

(2) A body which formed a compound with platinum chloride of the composition $(C_2H_7NO)_2\,(HCl)_2\,PtCl_4$, and to which he ascribed the formula



$$\left.\begin{matrix} C_2H_5O \\ H \\ H \end{matrix}\right\} N$$

This body could be regarded either as "dimethylamine, in which the third atom of H is replaced by (OH), or as oxyethylamine simply". This product is now generally referred to as amino-ethyl alcohol $NH_2 . CH_2 . CH_2 . OH$.

(3) A base whose platinum salt corresponded to the formula $C_5H_{14}N_2O . HCl . PtCl_4$.

Thudichum considered that the essential base of kephalin was choline, and that the amino-ethyl alcohol and the other base mentioned were merely secondary products formed from choline.

In the light of these results he regarded the constitution of kephalin as analogous to that generally accepted for lecithin. The principal kephalins always contained stearic acid, and for such a kephalin he suggested the formula :—

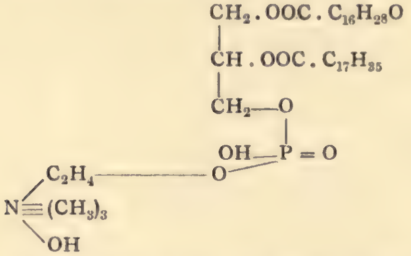
---

[1] In describing the base of both kephalin and lecithin Thudichum gives the correct formula for choline platinum chloride $(C_5H_{13}NO)_2\,(HCl)_2\,PtCl_4$. From this Thudichum deduced that the free base was represented by $C_5H_{13}NO$—the formula for Neurin. The reason for this is not very clear; probably he did not realise that these bases belonged to the type of ammonium hydroxide compounds but regarded them as containing trivalent nitrogen. In this monograph Thudichum's "neurin" is always referred to as choline.

Kephalin (Thudichum).

$$C_3H_5\left\{\begin{array}{l} C_{17}H_{28}O_3 \text{ Kephalic acid radicle.}\\ C_{18}H_{35}O_2 \text{ Stearic acid radicle.}\\ HO(OPO)C_5H_{12}NO. \end{array}\right.$$

This is similar to the lecithin formula already described, with the exception that kephalic acid is present here. This analogy is plainly brought out when the above formula is written like that given for lecithin [1] :—



Just as in the case of lecithin, Thudichum suggested an alternate formula in which the acid and glycerol radicles were directly attached to the phosphorus by substitution of the (OH) groups of phosphoric acid, "the base replacing an (OH) in glycerol, and being thus the earliest to be detached by hydrolysis".

Phosphoric Acid.

$$OP\left\{\begin{array}{l} OH\\ OH\\ OH \end{array}\right.$$

Kephalin.

$$OP\left\{\begin{array}{ll} C_{17}H_{28}O_3 & \text{Kephalic acid radicle.}\\ C_{18}H_{35}O_2 & \text{Stearic ,, ,,}\\ C_3H_7O_3 & \text{Glycerol radicle.} \end{array}\right.$$
$$\quad C_5H_{11}ON \text{ Choline ,,}^{1}$$

This hypothesis seemed to be substantiated by the fact that an intermediate product of hydrolysis was found which Thudichum called kephalophosphoric acid. This substance he considered to be composed of three acid radicles and one alcohol radicle.

$$OP\left\{\begin{array}{l} \text{Kephalic acid radicle.}\\ \text{Stearic ,, ,,}\\ \text{Glycerol radicle.} \end{array}\right.$$

This observation is of importance since different observers are agreed that complete hydrolysis of kephalin is carried out with great difficulty, the fatty acids tending to retain a considerable amount of phosphorus. Kephalin in this respect differs from lecithin which appears to be hydrolysed with comparative ease [Erlandsen, 1907].

While certain of Thudichum's observations with regard to the radicles of kephalin were found to be erroneous by subsequent investigators, his general conception of the nature of the constituents of this body proved to be correct. We now recognise that kephalin contains two fatty acids—one saturated and the other unsaturated (Thudichum's kephalic acid)—also a basic body (Thudichum's oxyethylamine) as well as glycerol and phosphoric acid: the latter two substances are perhaps combined as glycerophosphoric acid.

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[1] See footnote, p. 37.

Fatty Acids of Kephalin.

(*A*) *Saturated Fatty Acid.*

In addition to the ordinary stearic acid which Thudichum found in the principal kephalins, he claimed that other kephalins existed in small amount which did not contain stearic acid, but an acid probably homologous with stearic acid though of lower fusing-point, or an acid not homologous with stearic acid, and giving a lead compound soluble in ether. He thus postulated the existence of such varieties of kephalin as stearyl-kephalin, palmityl-kephalin and margyryl-kephalin. The source of Thudichum's kephalin is not quite clear; it was all obtained from brain and chiefly from human brain, but that ox brain was also used appears possible. Cousin [1906] subsequently investigated this problem of the fatty acids of kephalin, and succeeded in isolating only one saturated acid (stearic acid) from ox-brain kephalin, though he obtained evidence of the presence of traces of lower saturated acids.

Fränkel and Dimitz [1909], working with kephalin prepared from human brains, stated that the substance contained two homologous kephalins, one of which furnished stearic acid and the other palmitic acid; the palmityl-kephalin predominated. These conclusions of Fränkel and Dimitz are based chiefly on the result of analyses of a lead compound of high molecular weight which gave figures corresponding to a mixture of the two acids. Though it is claimed that these acids were actually obtained by fractional crystallisation from the mixture, no data beyond melting-points are furnished; obviously the melting-point of a body which has crystallised from a mixture of fatty acids cannot be regarded as any criterion of its purity unless substantiated by analytical figures. Further, as pointed out by Parnas [1909], oxyacids were probably present in the mixture. Thus the evidence for the conclusions of Fränkel and Dimitz is unsatisfactory. Parnas, in a very careful investigation of kephalin from human brain, isolated a fatty acid which on analysis proved to be pure stearic acid; he also showed that no other saturated acid was present. Later [1913] he again investigated the subject and reaffirmed his former results. Levene and West [1913, 2] came to similar conclusions; they examined four different specimens of brain kephalin and found only one saturated acid—stearic acid. At present, therefore, the evidence points to the conclusion that the only saturated acid found in kephalin prepared from human brain, is stearic acid. This acid has also been found by MacArthur and Burton [1916].

(*B*) *The Unsaturated Fatty Acid of Kephalin.*
Kephalic acid (*Thudichum*).
Kephalin linolic acid (*Parnas*).

Besides saturated fatty acids Thudichum recognised the presence of a special fatty acid in kephalin which he named "kephalic acid". Obviously the acid was obtained in an impure state and had probably undergone partial oxidation, but Thudichum indicated certain of its peculiarities, and concluded that it belonged to a series of acids containing at least one atom of oxygen more than the ordinary fatty acids. Analyses of its barium salts gave different formulæ, $C_{19}H_{31}O_3$, $C_{17}H_{30}O_3$ and $C_{17}H_{28}O_3$. Though in certain of its properties it failed to comply with the ordinary tests of purity, yet Thudichum regarded it as a chemical unit on account of the pertinacity with which it retained its properties and composition under most varied and severe treatment. The large amount obtained suggested that this body constituted an important part of the kephalin molecule. Thudichum, however, failed to recognise the essential nature of this acid, probably because of the oxidation changes which it had undergone, but concluded that it constituted the essential part of all kephalins. Kephalic acid, according to him, was the part of the kephalin molecule which bestowed on kephalin its characteristic properties. Kephalin might, therefore, be regarded as a lecithin in which one acid (oleic acid?) was substituted by the special kephalic acid. Besides kephalic acid Thudichum also obtained small amounts of another body, which was probably oleic acid though he did not recognise it as such.

Koch [1903] found that kephalin absorbed iodine, and concluded that it contained an unsaturated acid. This observation was confirmed by Cousin [1906, 1907], who estimated the iodine values of the fluid fatty acids obtained from kephalin. From the high iodine values found and the results of analyses Cousin identified the presence of an unsaturated acid containing two double bonds and belonging to the linolic acid series ($C_{18}H_{32}O_2$).

Falk [1908] oxidised the so-called kephalic acid with permanganate and obtained a small amount of a substance to which he gave the probable formula $C_{18}H_{36}O_4$.

Parnas [1909] partially hydrolysed some brain kephalin with baryta and isolated an intermediate product which contained acids and phosphorus, but was free from nitrogen. On further hydrolysis of this product with sodium hydroxide, a yellow oil was ultimately obtained, constituting about 18 per cent. of the original kephalin. This substance was transformed into the methyl ester, which on saturation with hydrogen in the presence of palladium gave the methyl ester of stearic acid melting at 69°. This result proved that the unsaturated acid present belonged to the C_{18} series. On saponification of the unsaturated methyl ester a light yellowish oil was obtained, which on analysis gave the formula $C_{18}H_{32}O_2$ (linolic acid). On saturating this acid with hydrogen, stearic acid melting at 68° was obtained. These results leave no doubt whatever that the chief unsaturated acid of brain kephalin is identical with, or similar to, linolic acid with which it agrees in composition and properties. It is possible, however, that this kephalin linolic acid may be a mixture of stereoisomers of linolic acid.

The statement of MacArthur and Burton [1916] that oleic acid constitutes about 50 per cent. of the total fatty acids of kephalin is not in agreement with the results of any other investigator.

These observers also found indications of the presence of other acids which they suggested might be cephalinic and clupanodonic acids: they admit, however, that the evidence for the presence of these acids is inconclusive. The presence in kephalin of very small amounts of oleic acid, and an acid with three unsaturated double bonds (similar to the cephalinic acid of MacArthur and Burton), was shown by Parnas [1909], but they probably arose from impurities in the kephalin. Parnas concluded that the two chief acids of kephalin are stearic and kephalin linolic acids.

The Base of Kephalin.

Thudichum's assumption that choline was the essential base of kephalin, and that the other bases found by him (amino-ethyl alcohol and a third ill-defined body) were decomposition products of choline, has been proved to be wrong. In a preparation of kephalin from sheep's brain Koch [1902] could obtain no evidence of choline. On the contrary he isolated from the products of hydrolysis a small amount of a platinum salt which seemed to be a monomethyl-oxyethyl-amine compound; unfortunately the quantity of substance at his disposal was too small for further purification. As the result of treating kephalin with hydriodic acid, he obtained evidence that the base was a methyl compound in which each nitrogen atom was attached to only one methyl group. It would appear, however, that Koch's results were due to the presence of glycerol in the molecule, and did not really give any information regarding the nature of the base. Cousin [1907], working with ox brain kephalin, found choline and no other base. He also took the view that Thudichum's other bases were decomposition products. Fränkel and Neubauer [1909], who carried out investigations on three different specimens of kephalin, were unable to find any choline, but substantiated Koch's conclusion that kephalin contained one methyl group attached to each atom of nitrogen. Consideration of their experimental data hardly seems to justify their conclusion.

The subject was then investigated by Parnas and his co-workers [Baumann, 1913; Renall, 1913], who definitely showed that the base of kephalin is amino-ethyl alcohol ($NH_2 . CH_2 . CH_2 . OH$), and that there is no evidence of the presence of another substance containing nitrogen. These investigators showed that all the nitrogen of kephalin exists in the form of a primary amino base, and could be quantitatively estimated by the method described by Van Slyke [1912].[1] The base was also isolated in the form of the gold salt and identified by analysis. MacArthur [1914] claimed to have shown that amino-ethyl alcohol accounted for but a comparatively small amount of the total nitrogen of kephalin. According to this observer kephalin nitrogen is present in four forms:—

(1) Ammonia nitrogen = 0·2 per cent.[2]
(2) Residual ,, = 0·2 per cent.
(3) Amino-ethyl alcohol nitrogen = 0·8 per cent.
(4) Amino acid nitrogen = 0·4 per cent.

The large amount of amino acid nitrogen found is striking, but before accepting these figures further work will be necessary.

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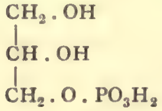
[1] See Plimmer's "Chemical Constitution of the Proteins". Part I, Analysis. 1917.

[2] The percentages given are calculated on the weight of kephalin taken. They add up to 1·6 per cent., which roughly represents the percentage of nitrogen in kephalin.

Glycerol and Phosphoric Acid of Kephalin.

The presence of glycerol in kephalin has been accepted by all observers, though its identification has apparently been arrived at in an indirect manner. There appears to be some doubt whether the relationship between glycerol and phosphorus in the kephalin molecule is similar to that found in lecithin, in which glycerophosphoric acid is undoubtedly present. Thudichum claims to have isolated glycerophosphoric acid from kephalin, but as his kephalin was obviously impure, as indicated by the presence of considerable amounts of choline, no great reliance can be placed on this result. The same observation applies to Cousin's statement [1907] that the glycerophosphoric acid in kephalin is identical with that of lecithin. The results of Fränkel and Dimitz [1909], who worked with a purified choline-free specimen of kephalin, support the view that glycerophosphoric acid is present. These observers obtained from kephalin a barium salt, which had the general properties of barium glycerophosphate, and contained practically the theoretical amount of barium. The composition of this salt was given as $C_3H_7O_6PBa + H_2O$, though the only analytical figures furnished refer to the barium content. Since the barium salt was optically active they ascribed to the corresponding acid the asymmetrical formula

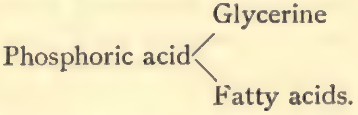


$$\begin{array}{l} CH_2 . OH \\ | \\ CH . OH \\ | \\ CH_2 . O . PO_3H_2 \end{array}$$

just as Willstätter and Lüdecke [1904] did for the corresponding lecithin compound. This barium compound was found to be *dextro*-rotatory, and contained *one* molecule of water of crystallisation. It differed therefore from the corresponding lecithin salt isolated by Willstätter and Lüdecke, which was *laevo*-rotatory, and contained only *one-half* molecule of water. Unlike the glycerophosphoric acid of lecithin it was very resistant to acid hydrolysis.

The results of Fränkel and Dimitz are the strongest direct indication we have of the occurrence of glycerophosphoric acid in kephalin, and it must be admitted that they support the probability that this acid is actually present. On the other hand, the analytical data furnished are not sufficiently extensive to settle the matter beyond dispute, and Parnas [1909] considers that the presence of glycerophosphoric acid in kephalin has not been definitely established.

The idea that ordinary glycerophosphoric acid may not constitute part of the kephalin molecule is apparently supported indirectly by several observations. When, for instance, kephalin is hydrolysed, it is exceedingly difficult to get phosphorus-free fatty acids. In fact, definite intermediate compounds containing fatty acids and phosphorus have been obtained, particularly by Thudichum and Parnas. Thudichum's substance, kephalo-phosphoric acid, has been already referred to. The product obtained by Parnas was a tetrabasic body corresponding to the formula $C_{27}H_{53}O_{10}PBa_2$. This product was isolated after the kephalin had been autoclaved for twelve hours at 120° with barium hydrate; on further hydrolysis with sodium hydroxide this substance was found to contain a considerable amount of linolic acid, but apparently no stearic acid. If this compound can be accepted as a chemical unit it would appear that the linolic acid of kephalin must be in intimate relationship with the phosphorus atom, while the stearic acid is split off with comparative ease. In lecithin, on the other hand, both acids are easily separated by hydrolysis, and no corresponding intermediate products have been isolated. This suggests that the structure of kephalin differs from that of lecithin where both fatty acids are attached to glycerol. In kephalin one of the acids at any rate seems to be directly attached to the phosphoric acid group, and though glycerophosphoric acid might still be present in such a combination, it is difficult to suggest a formula to satisfy the facts. On the whole, the observations made on the hydrolysis of kephalin, and the presence of intermediate bodies, suggest for this substance a structure based on the lines indicated by Thudichum, where the acids are directly attached to the phosphoric acid group.



This, however, leaves many of the difficulties unexplained, but appears preferable to the structure in which kephalin is represented as a body similar to lecithin but containing different acids and a different base.

The Composition and Elementary Analysis of Kephalin.

According to the above observations the kephalin molecule probably consists of:—

$$\text{(1) Two Fatty acids}\begin{cases}\text{Stearic } C_{18}H_{36}O_2\\ \text{Linolic } C_{18}H_{32}O_2\end{cases}$$

(2) A base—amino-ethyl alcohol, $NH_2 . CH_2 . CH_2 . OH$

(3) Glycerol and phosphoric acid,

but at present we can say nothing with certainty as to the manner in which the different constituents are combined. Assuming that each of these substances is present in the kephalin molecule in equimolecular proportions, and that glycerol and phosphorus are combined as glycerophosphoric acid, kephalin should have the following formula:—

$C_{41}H_{78}NPO_8$ C = 66·17 per cent.
H = 10·57 ,,
N = 1·88 ,,
P = 4·17 ,, with a N : P ratio of 1 : 1.

A comparison of the theoretical value with those actually obtained by various observers shows that a considerable discrepancy exists, particularly with regard to the percentage of C and H. In such a large molecule as that of kephalin no great weight can be attached to small differences in these figures, but here, even in cases of very carefully purified kephalin, the difference is greater than can reasonably be accounted for by experimental error.

The chief results obtained by various investigators are seen in the table.

Investigator.	Source of Kephalin.	C.	H.	N.	P.	N : P Ratio.
Thudichum [1884]	Brain (human and ox)	60·00	9·38	1·68	4·27	1 : 0·87
Zuelzer [1899]	,, (ox)	60·20	9·80	3·80	2·60	1 : 0·32
Koch [1902]	,, (sheep)	59·50	9·80	1·75	3·85	1 : 1·01
Cousin [1906]	,,	—	—	1·82	3·89	1 : 1·04
Stern and Thierfelder [1907]	Egg yolk	59·68	9·74	1·57	3·64	1 : 0·96
,, ,, ,, ,,	,, ,,	65·66	11·54	1·37	3·96	1 : 0·77
Falk [1908]	Nerves	55·75	9·66	1·94	4·42	1 : 0·97
,, ,,	Brain (human)	57·56	9·21	2·93	3·23	
Fränkel and Neubauer [1909]	,, ,,	61·99	9·85	1·65	3·44	1 : 1·06
		62·12	9·87	1·69	3·45	1 : 1·08
Parnas [1909]	,, ,,	—	—	1·83	3·86	1 : 1·05
Frank [1913]	Liver	57·10	9·62	1·72	3·91	1 : 0·97
Baumann [1913]	Brain (human)	—	—	1·84	—	—
Renall [1913]	,, (ox)	—	—	1·69	3·56	1 : 1·05
	,, (sheep)	—	—	1·92	4·27	1 : 1·00
Bürger and Beumer [1913]	Erythrocytes	—	—	1·78	4·06	1 : 0·97
Levene and West [1916]	Brain	60·00	9·62	1·78	3·60	1 : 1·09
,, ,, ,, [1916, 2]	Liver	60·33	9·01	1·86	3·75	1 : 1·10
,, ,, ,, ,,	Kidney	60·17	8·95	1·70	3·65	1 : 1·03
,, ,, ,, ,,	Egg yolk	60·00	9·62	1·78	3·69	1 : 1·07

From these figures it appears that the carbon actually found is at least 5 per cent. less than the theoretical requirements, while the percentages of the other elements are also low. In fact, as pointed out by Levene and West [1916], the formula $C_{41}H_{78}NPO_{13}$ agrees much better with the analytical figures found than does the calculated $C_{41}H_{78}NPO_8$. Thudichum realised this difficulty, and endeavoured to account for the difference by assuming that each molecule of kephalin contained 5 molecules of H_2O. His kephalin gave analytical figures corresponding to the formula $C_{42}H_{79}NPO_{13}$, which on deduction of 5 molecules of H_2O = $C_{42}H_{69}NPO_8$. This latter formula agrees well with the theoretical one except that the hydrogen content is much too low. Thudichum observed that the assumption that kephalin took up 5 atoms of oxygen would give analytical numbers agreeing with those actually found, but he rejected this explanation on theoretical grounds.

The apparent excess of oxygen, as ascertained by analysis, is at least partly accounted for by oxidation of the unsaturated linolic acid present in the kephalin.

It appears probable that every sample of kephalin hitherto analysed had undergone oxidation to a greater or less extent during the process of preparation. Theoretical figures cannot be expected from analysis, while the substance is prepared by the methods at present in vogue. It is not impossible that the particular unsaturated acid present may be an isomer, with a more marked tendency to oxidation than the ordinary linolic acid.

An iodine value carried out immediately before elementary analysis would furnish some evidence of the amount of oxidation which had taken place in the molecule. That certain samples of kephalin had the greater part of their unsaturated acid oxidised before analysis was carried out, is obvious from the description of the methods used in their preparation.

If, for the sake of argument, we assume the whole of the unsaturated acid in a sample of kephalin to be fully oxidised, this would mean the addition of four (OH) groups to the calculated formula:—

$$C_{41}H_{78}NPO_8 + 4(OH) = C_{41}H_{82}NPO_{12}.$$

Now $C_{41}H_{82}NPO_{12}$ gives C = 60·96, H 9·66, N 1·73, P 3·84, a percentage result which is in reasonably close agreement with the majority of the results obtained by different observers.

On the other hand, the analyses indicate high oxygen figures in many samples of kephalin, where precautions against oxidation had apparently been adopted, but the unsaturated state of the molecule, as shown by iodine value or hydrogen absorption capacity, is often not indicated: there is therefore no criterion as to what extent these precautions had been successful in attaining their object.

When kephalin is treated with hydrogen in the presence of palladium or other suitable catalyst, each molecule should absorb four atoms of hydrogen, provided no oxidation of the unsaturated radicle had previously taken place. Levene and West [1916] have attempted to purify kephalin by hydrogenising the molecule and separating the product formed, but so far without much success. These observers obtained kephalin which, judging from the various manipulations it had undergone at high temperatures, had at least a good chance of being partially oxidised; this kephalin corresponded to the formula $C_{41}H_{78}NPO_{13}$. Unfortunately, they do not state whether the actual sample which they hydrogenised was part of the above or corresponded to this formula. If the specimen actually used for hydrogenisation had the formula $C_{41}H_{78}NPO_{13}$, the fact that it absorbed practically the theoretical amount of hydrogen excludes the possibility of oxidation of the unsaturated acids being the cause of the high oxygen content; indeed a specimen oxidised to this extent should absorb little or no hydrogen. Levene states that certain of his samples did actually absorb little or no hydrogen; while this might be due to traces of impurities, the statement is made that further attempts to purify the kephalin did not increase the absorption.

At present the available evidence tends to suggest that the difference between the calculated values and those actually obtained in kephalin analyses is largely due to oxidation of the unsaturated acid in the molecule, but since the evidence itself is unsatisfactory the question must be left an open one.

The extent to which oxidation is responsible for this discrepancy might be ascertained by careful correlation of the iodine value, the analytical figures and the hydrogen absorption capacity of any given specimen.

It is also probable that the properties of kephalin are such as to preclude the preparation of a chemically pure specimen by the methods hitherto adopted. Levene rejects this view on the ground that lecithin, which closely resembles kephalin in its physical and chemical properties, gives analytical values, which are in accordance with the constitution usually ascribed to lecithin. Reference to the discussion on the composition of lecithin shows that any agreement there may be is purely accidental, since the lecithin furnishing the figures consisted of two different bodies.

Levene and West [1916] endeavoured to estimate the percentage of the different components of kephalin present in the molecule, and obtained the following results:—

	Found.	Calculated for $C_{41}H_{78}NPO_8$.
Base	8·26	8·2
Glycerol	10·00	12·4
Phosphoric acid	12·17	13·2
Fatty acids	63·40	75·9
Totals	93·83	109·7

There is a marked deficiency in the fatty acids which is difficult to account for. In obtaining the acids the kephalin was boiled with 3 per cent. H_2SO_4 for twenty-four hours, but since kephalin is very resistant to hydrolysis, and specimens hydrolysed for twenty-four to thirty-five hours with 5 per cent. hydrochloric acid "still seemed to contain unchanged kephalin" [Fränkel and Dimitz, 1909], it is just possible that the whole of the acids were not split off under the conditions of the experiment, though this does not appear very probable.

All the difficulties with regard to the analysis of kephalin may be due, as suggested by Levene, to the presence in the kephalin molecule of some substance hitherto undiscovered. It is open to question, however, whether such disturbing factors as those mentioned—oxidation, the tendency to decomposition, the difficulty of preparing a pure substance and the general unreliability of the C and H percentages as ascertained in a large molecule like kephalin—may not account for the discrepancies.

Much future research will be required to settle these points, and in the meantime no definite answer can be given to the question as to whether the prevailing hypothesis of the structure of kephalin is correct. Levene's careful experiments which are still being carried out should help to settle the point. It seems doubtful, however, whether a pure specimen of kephalin has ever been prepared.

Some Kephalin-like Substances (Thudichum).

Besides ordinary kephalin Thudichum [1884] described certain other allied substances which he claimed to have isolated from brain. These bodies show a marked similarity with kephalin, both in elementary composition and general properties. From our knowledge of kephalin it is almost certain that Thudichum's substances were oxidised kephalins, which in many or all cases contained impurities as well.

Thudichum mentions :—

Amidokephalin	$C_{126}H_{239}N_5P_3O_{39}$
Oxikephalin (as $CdCl_2$ compound)	$C_{42}H_{79}NPO_{14} . CdCl_2$
Peroxikephalin	$C_{42}H_{79}NPO_{15}$
Kephaloidin	Same as kephalin (but different properties)
Oxikephaloidin (as $CdCl_2$ compound)	$2(C_{42}H_{75}NPO_{14})CdCl_2$

Many of these substances were prepared from specimens of crude kephalin, which had not undergone the ordinary processes of purification used by Thudichum in preparing kephalin.

Chief Properties of Kephalin.

The chief properties of kephalin have been described by Thudichum [1884], Falk [1908], Fränkel and Neubauer [1909] and Parnas [1909].

Kephalin as usually obtained is a hard yellowish-brown somewhat brittle substance, which can be easily pulverised; it is very hygroscopic and in the presence of moisture forms a sticky tenacious mass. The colour of kephalin is due to impurities; Parnas isolated a specimen which was colourless. Kephalin has not been obtained in crystalline form. From very cold ether solution it separates in the form of doubly-refracting globules, which show no definite structure (Parnas).

According to Thudichum, kephalin "when not too much hydrated" is highly soluble in ether, and when dry is soluble in anhydrous ether in almost any proportions. This statement of Thudichum appears to be incorrect, for Parnas found that kephalin is insoluble in *anhydrous* ether, but dissolves readily in ether containing 1 per cent. of water, an observation which has been corroborated by Levene and West [1916]. Kephalin dissolves also in chloroform, benzene, petroleum ether and carbon disulphide: in cold acetic ether it is practically insoluble, but dissolves on heating and falls out on cooling: like other phosphatides it is insoluble in acetone.

With regard to the behaviour of kephalin towards alcohol, various contradictory statements have been made. Thudichum's kephalin was slightly soluble in cold alcohol and fairly soluble in hot alcohol, while Koch's preparation was quite insoluble in alcohol, though the presence of a small amount of hydrochloric acid rendered it fairly soluble. There seems no doubt that kephalin is really somewhat soluble in alcohol, but the degree of solubility may depend on the treatment adopted in its isolation. Thus Fränkel and Neubauer found that kephalin prepared by alcohol precipitation was quite insoluble in this reagent, but that the same sample of kephalin when purified by means of hydrochloric acid was easily soluble: on treatment with acetone it again became insoluble.

Since kephalin usually contains bases such as calcium and potassium, an explanation of this action of hydrochloric acid was sought in the supposition that ordinary alcohol-insoluble kephalin was really a compound from which free kephalin was liberated on treatment with acid. In this connexion Bang [1911] suggests the probability that free kephalin is really alcohol soluble, but this seems to be discounted by the action of acetone on the soluble kephalin.

With water, kephalin forms suspensions and ultimately clear colloidal solutions. From its aqueous solution kephalin is precipitated by treatment with hydrochloric acid and with various inorganic and organic acids. Tartaric acid does not produce this effect. This separation does not depend on the formation of compounds of kephalin with acids as Thudichum suggested, but is apparently a physical reaction in which the negatively charged kephalin is precipitated by the positively charged acid [Fränkel and Neubauer, 1909]. Kephalin may also be obtained from colloidal solutions by centrifuging [Parnas, 1909].

By the action of water, acids or alkalies, kephalin is partly decomposed, the base and part of the fatty acids being split off with great ease; on the other hand, complete hydrolysis of kephalin is not easily accomplished, even on boiling with acids or alkalies for considerable periods. On exposure to air it readily absorbs oxygen, a property dependent on the unsaturated fatty acid of the molecule. Solutions of kephalin in ether are generally characterised by a marked fluorescence, which according to Parnas depends on the interaction of salts with the earlier decomposition products of kephalin. When an ethereal solution of kephalin is shaken with hydrochloric acid, the fluorescence disappears, but returns on the addition of a small amount of lime water to the washed ethereal solution.

Kephalin is very hygroscopic and has such a marked affinity for water that the expulsion of the last trace of moisture is obtained only after long treatment *in vacuo* (Thudichum).

According to Parnas [1909] and Falk [1908] the melting-point of kephalin is 174°. Fränkel's specimen melted at 175°.

Kephalin always contains inorganic constituents such as calcium, potassium and ammonia: Parnas found traces of magnesium as well, but could not confirm the statement of Falk that iron and copper were also present.

According to Fränkel and Neubauer, kephalin is optically active and rotates the plane of polarised light to the right. In one experiment 0·11 gm. of kephalin dissolved in 10 c.c. petroleum ether and examined in a tube 1 decimetre long gave a rotation of 0·15° to the right; for this concentration the result indicates a specific rotation of +13·6°.

Compounds of Kephalin.

Kephalin forms compounds with cadmium chloride and platinum chloride, but these combinations are not well characterised. For the cadmium chloride compound Thudichum obtained on analysis 89·38 per cent. kephalin and 10·62 per cent. cadmium chloride, while theory requires 17·97 per cent. cadmium chloride. For the platinum chloride combination the same observer found 3·6 per cent. platinum chloride against the theoretical 9·5 per cent. Both these combinations are soluble in ether but insoluble in alcohol. From its aqueous colloidal solution kephalin is precipitated by many salts, alkalies and other reagents [Thudichum, 1884; Koch, 1903, 1907], but many of these precipitates are not true kephalin compounds, but result from the physical action of these substances on the colloidal kephalin solution. Kephalin appears to form a definite compound with lead acetate in the presence of ammonia, a reaction which is often used in its isolation and purification.

The subject of kephalin combinations is in an unsatisfactory condition and requires further investigation. Levene and West [1916, 3] mention phenyl and naphthyl compounds of kephalin.

CUORIN.

In the ether extract of heart muscle Erlandsen [1907] found a phosphatide, which was insoluble in alcohol and was at first thought to be kephalin. It differed, however, from kephalin in having two atoms of phosphorus in the molecule and on this account Erlandsen recognised it as a new monamino-diphosphatide and called it cuorin (Ital. cuore = heart).

Cuorin has not yet been fully investigated, and while there appears to be substantial evidence that the substance is a chemical unit, it is not impossible, on the other hand, that the body is simply kephalin contaminated with a phosphorus compound. When prepared from products obtained in the course of the preparation of phosphatides by the ordinary methods at present in vogue, the body must be mixed with kephalin. The removal of kephalin or other impurities may under certain conditions be brought about by treatment with hot alcohol [Erlandsen, 1907], but there are other cases in which this may not be possible. For reasons, which will be given later, it is obvious that in all attempts to obtain this substance, the necessary manipulations must be carried out with the greatest expedition, while purity of reagents and protection from oxygen are essential.

The presence of cuorin in other tissues besides heart is highly probable. Thus MacLean [1912, 2] isolated a substance from horse kidneys which in properties and elementary composition agreed with cuorin. Cuorin-like substances were also isolated from liver [Baskoff, 1908] and from egg yolk [MacLean, 1909, 2]. Baskoff's substance which he called "heporphosphatide" had a N : P ratio of 1 : 1·5, which seems to indicate the probability that the substance was a mixture of cuorin with kephalin. The body obtained from egg yolk had a N : P ratio of exactly 1 : 2, but differed considerably from cuorin in its nitrogen and phosphorus percentage. As suggested by Bang [1911] it may have been a decomposition product due to the somewhat drastic treatment with hot alcohol employed in its isolation. At present the only well-characterised cuorin is that of heart, but even this may be found to be a mixture. With regard to the other tissues, all that can be said with certainty is that some of them contain small amounts of a body closely resembling kephalin in physical and chemical properties, but having considerably more phosphorus than kephalin. Whether these bodies are mixtures, or, in some cases, artificial products, is at present undecided

Structure of Cuorin.

The nature of the structural formula of cuorin has not yet been suggested by any observer. Erlandsen found that his cuorin contained in the molecule three fatty acids, glycerol (probably in combination with phosphoric acid as glycerophosphoric acid) and a base which he failed to identify but which was not choline.

The fatty acids present belonged wholly or in part to the unsaturated series, and judging from their iodine values linolic acid $C_{18}H_{32}O_2$ appeared to be the chief constituent, though linolenic acid $C_{18}H_{30}O_2$ may also perhaps have been present. In three hydrolysis experiments the percentages of fatty acids obtained from cuorin amounted to 64·40 per cent., 63·62 per cent. and 63·74 per cent.

Elementary Analysis of Cuorin.

The elementary analysis of cuorin gives for hydrogen and carbon practically the same results as kephalin, while the nitrogen and phosphorus figures differ considerably from those of kephalin. The different preparations of cuorin analysed by Erlandsen gave very consistent results, as seen in the table, where the figures obtained for certain cuorin-like bodies are also given.

Observer.	Source of Material.	C.	H.	N.	P.	N : P Ratio.
Erlandsen [1907]	Ox heart	61·86 61·46 61·57	8·93 8·92 9·11	1·01 1·02 —	4·43 4·41 —	1 : 2
,,	,,	61·76 61·52	9·18 9·09	1·01 1·03	4·46 4·52	1 : 2
,,	,,	— —	— —	— 1·01	4·46 4·50	1 : 2
MacLean [1912, 2]	Horse kidney	60·4	10·2	1·04	4·45	1 : 2
,, [1909, 2]	Egg yolk	59·12	9·44	0·812	3·59	1 : 2
Baskoff [1908]	Liver	61·12	8·95	1·23	4	1 : 1·5

Chief Properties of Cuorin.

The chief physical and chemical properties of cuorin are the same as those of kephalin. Cuorin is a golden-brown transparent substance, which on drying becomes hard and easily pulverised. It is exceedingly hygroscopic and on addition of water becomes sticky and ultimately fluid. It readily oxidises on exposure to the air, and in about a month takes up oxygen to the extent of about 9 per cent. of its weight. Cuorin is easily soluble in ether, chloroform, petroleum ether and carbon disulphide; it also dissolves in benzene, but not readily at ordinary temperature. On heating it is also soluble in ethyl acetate, glacial acetic acid and amyl alcohol, but is precipitated from solution on cooling. With water, cuorin forms an emulsion which becomes clear on addition of an alkali. In acetone it is insoluble.

When oxidised, cuorin is much harder than in the fresh state, and tends to become insoluble in ether and other organic solvents, while it dissolves completely in water.

Cuorin forms combinations with platinum chloride and cadmium chloride. A sample of freshly prepared cuorin gave an iodine value of about 101, while an oxidised specimen gave only 22.

While cuorin is described here as a chemical unit with all reserve, the evidence available at present suggests that cuorin does exist in heart and kidneys and perhaps in other tissues. There does not appear to be any definite proof that the substance is not a mixture, so that the question must of necessity be left open.

SPHINGOMYELIN.

When brain tissue is extracted with warm alcohol and the alcohol extract concentrated and cooled, a white powdery substance separates in the form of a precipitate. This precipitate is composed largely of sphingomyelin and cerebrosides. Sphingomyelin, which was first isolated by Thudichum [1883], differs from the phosphatides already described in being a white crystalline stable substance. It is very abundant in the brain, but occurs to some extent in many other tissues, and is always found in association with cerebrosides, with which it has many properties in common; on this account its separation in pure form is exceedingly difficult, and nearly all the sphingomyelins described have been contaminated with cerebrosides to a greater or less degree. These mixtures of sphingomyelin and cerebrosides (often referred to as "*Protagon*") have hitherto given rise to much confusion, but modern work has practically removed the difficulties. This aspect of the subject is fully discussed in the chapter on protagon.

Thudichum recognised that sphingomyelin was a diaminomonophosphatide (N : P = 2 : 1); he showed that it differed from other phosphatides in containing no glycerol.

As the result of hydrolysis of various specimens of impure sphingomyelin with barium hydroxide, Thudichum obtained the following products:—

(1) Phosphoric acid.
(2) A barium salt soluble in ether.
(3) A fatty acid.
(4) An alcohol—sphingol.
(5) Two bases—sphingosine and neurine (choline, see footnote, p. 37).

(1) *Phosphoric Acid of Sphingomyelin.*

This was always obtained free and never in combination with glycerol as in the case of lecithin and other phosphatides. Thudichum therefore came to the conclusion that sphingomyelin contained no glycerol, an observation which is now accepted by all observers.

(2) and (3) *Thudichum's Ether-soluble Barium Salt and Fatty Acid.*

The barium salt might probably have been the salt of a fatty acid, but Thudichum furnished no data with regard to its composition. He obtained, however, a free fatty acid whose barium and lead salts were insoluble in ether and in alcohol. This acid corresponded to the formula $C_{18}H_{36}O_2$. While agreeing with stearic acid in its elementary analysis it melted at 57°, thus differing from stearic acid which melts at 69°. Thudichum considered this acid to be an isomer of stearic acid and on this account he named it sphingostearic acid.

(4) *Sphingol.*

This substance, to which Thudichum gave the name sphingol, corresponded to the formula $C_9H_{18}O$ or $C_{18}H_{36}O_2$. Assuming the latter formula to be the correct one, the body would constitute an isomer of stearic acid. It is very doubtful, however, whether Thudichum's sphingol exists.

(5) *Sphingosine.*

Sphingosine was first isolated by Thudichum who, from analyses of its salts, correctly ascribed to it the formula $C_{17}H_{35}NO_2$. From its behaviour towards acids and bases Thudichum considered the possibility of the substance having the structure of an amino acid, and observed that it might possibly be regarded as an acid of the $C_nH_{2n}O_2$ series in which 1 atom of hydrogen was replaced by an amino group. The composition of such a substance would be expressed by the formula $C_{17}H_{33}(NH_2)O_2$. On the other hand, since the body had great affinity for acids and was precipitated by most of the specific reagents for alkaloids, Thudichum considered that, on the whole, the evidence pointed to the conclusion that sphingosine was an alkaloidal base.

Sphingosine was next found by Thierfelder [1904] who in conjunction with his pupils independently established the formula $C_{17}H_{35}NO_2$ for this substance. This formula agreed with Thudichum's, but it is interesting to note that Thierfelder's early investigations were carried out in ignorance of Thudichum's work. Thierfelder also showed that sphingosine was a monacidic base yielding stable salts with mineral acids [Thierfelder, 1904, 1905; Kitagawa and Thierfelder, 1906].

After 1906 no important contribution to the chemistry of sphingosine appeared until the end of 1911, when Levene and Jacobs [1911, 1912] showed that sphingosine was a dihydroxy-derivative of a primary unsaturated amine. About the same time Riesser and Thierfelder [1912] and Thomas and Thierfelder [1912] furnished independent evidence to the same effect. The principal reactions of sphingosine on which these statements were based are as follows [Levene and Jacobs, 1912]:—

1. Sphingosine gives up the whole of its nitrogen on treatment with nitrous acid by Van Slyke's method. The nitrogen must therefore have been present as a primary NH_2 group.
2. Sphingosine readily absorbs hydrogen on treatment by the method of Paal and forms a dihydrosphingosine, thus indicating the presence of a double bond.

$$\underset{\text{Sphingosine}}{C_{17}H_{35}NO_2} + H_2 = \underset{\text{Dihydrosphingosine.}}{C_{17}H_{37}NO_2}$$

3. On acetylation a *tri*acetyl derivative is formed which no longer contains the primary amino group. The amino group has therefore been responsible for one acetyl group; the other two indicate the presence of two (OH) groups.

So far it was proved that sphingosine contained two (OH) groups, one primary (NH_2) group and a double bond, but nothing was known with regard to their position in the molecule. There was also no knowledge as to whether the carbon atoms were linked together in a normal or branched chain.

On treatment of sphingosine with oxidising agents, oxidation should take place at the double bond or possibly a carbon atom attached to an amino or hydroxyl group might be oxidised. On careful oxidation with chromium trioxide, Lapworth [1913] obtained a normal 13-carbon or tridecylic acid, and for this reason he concluded that the carbon atoms of sphingosine were linked together in a straight chain. This observation of Lapworth limited the position of the amino and hydroxyl groups to the four terminal carbons of the chain and rendered it highly probable that the double bond existed between the thirteenth and fourteenth carbon atoms:—

$$CH_3(CH_2)_{11} . CH = CH . C_3H_4(OH)_2(NH_2).$$

Lapworth's results were confirmed and extended by Levene and West [1914; 1914, 2], who saturated sphingosine with hydrogen by Paal's method and oxidised the dihydrosphingosine formed. In this case cleavage took place not between the thirteenth and fourteenth carbon atoms but between the fifteenth and sixteenth, with the formation of a 15-carbon acid which was identified as normal pentadecylic acid.

The cleavage of sphingosine described by Lapworth must therefore have taken place at the double bond since saturation of the unsaturated linkage prevented oxidation at this point. The formation of pentadecylic acid also further limited the position of the (OH) and (NH_2) groups to the three terminal carbon atoms. The formula of sphingosine may therefore be expressed approximately as follows :—

$$\underbrace{\underbrace{CH_3 . (CH_2)_{11}CH}_{\substack{\text{Tridecylic acid on}\\ \text{oxidation}}} = CH . CH(OH)}_{\substack{\text{Pentadecylic acid on oxidation of}\\ \text{saturated base.}}} . CH(OH) . CH_2(NH_2)$$

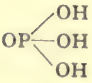
Attempts to determine the distribution of the amino and hydroxy groups on the last three carbon atoms have been made by Levene and Jacobs [1912], who endeavoured to reduce dihydrosphingosine to the corresponding amine. The normal heptyldecamine having the composition $CH_3 . (CH_2)_{16} . NH_2$ has been obtained synthetically, and comparison of the two bodies should determine whether or not they are similar. If they were found to be identical the composition of sphingosine would be settled, but so far attempts to reduce dihydrosphingosine to the corresponding amine have proved unsuccessful, an unsaturated amine being obtained.

A description of various salts and derivatives of sphingosine is given by Levene and West [1916, 1].

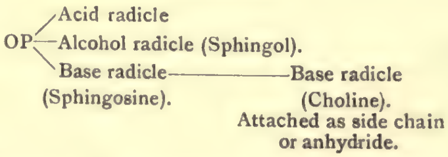
Composition and Structure of Sphingomyelin.

Since free phosphoric acid was always obtained on hydrolysis and glycerophosphoric acid was absent, Thudichum suggested that the composition of sphingomyelin might be represented by a formula in which the three (OH) groups of phosphoric acid were substituted by an acid, alcohol and basic radicle, the other base being attached as a side chain, or anhydride, to a radicle already substituted.

Phosphoric Acid.



Sphingomyelin.



Sphingomyelin (Thudichum's actual formula).

$$OP\begin{cases} C_{18}H_{35}O_2 & \text{(Stearic acid).}\\ C_{18}H_{35}O_2 & \text{(Sphingol).}\\ C_{17}H_{34}O_2N & \text{(Sphingosine).}\\ C_5H_{11}N & \text{(Neurine).}\end{cases}$$

In the main Thudichum's general observations on sphingomyelin have been confirmed by subsequent research, though in several points of detail he was obviously in error.

Sphingomyelin was investigated by Rosenheim and Tebb [1908; 1909, 1; 1910, 1], who, using a new method, isolated what they considered to be a fairly pure specimen. The general properties of this substance agreed with those given by Thudichum, and on hydrolysis it yielded choline and fatty acids, but no glycerine. On partial hydrolysis a body was isolated which had some resemblance to the simplest nucleic acids; on complete hydrolysis this intermediate product yielded phosphoric acid, a base and a crystalline alcohol. With regard to the structural composition of sphingomyelin, Rosenheim and Tebb expressed no opinion.

Levene [1913] showed that one of the hydrolytic products of sphingomyelin was an acid which melted at 81°. This acid was identified as lignoceric acid $C_{24}H_{48}O_2$ (see p. 97, also Leathes, 1910).

All the specimens of sphingomyelin investigated up to this point were impure and contained cerebrosides, but Levene [1914] reported a method for obtaining pure sphingomyelin and ascertained the best conditions for the hydrolysis of this substance. Working with pure specimens Levene [1916] obtained the following products on hydrolysis:—

1. Phosphoric acid.
2. Two fatty acids; (*a*) Lignoceric acid $C_{24}H_{48}O_2$; (*b*) An acid of a low melting-point which could not be obtained pure, but which was probably a hydroxy acid; it constituted at least 50 per cent. of the acids of sphingomyelin.
3. Two bases: (*a*) Choline $C_5H_{15}NO_2$; (*b*) Sphingosine $C_{17}H_{35}NO_2$.

Another base corresponding to the constitution[1] $C_{17}H_{33}NO$ (sphingosine minus H_2O) was found, but evidence was obtained that this base was a secondary product formed from sphingosine.

When sphingomyelin is hydrolysed with alcohol and water, sphingosine loses a molecule of water giving rise to a base of the nature of anhydrosphingosine. This secondary product must contain two unsaturated linkages, one of them being in the position of the double bond in sphingosine. The relation of this body to sphingosine will be at once apparent on reference to the structural formula of the latter substance. The (OH) and (H) of sphingosine shown in heavy type are supposed to be eliminated as water. The position of the second double bond in the resulting substance is arbitrary :—

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[1] When giving the empirical formula of this base Levene constantly refers to it as $C_{17}H_{35}NO$. It is probable that this is a slip, as the formula $C_{17}H_{33}NO$ represents sphingosine from which one atom of oxygen has been withdrawn, and it is definitely stated that this secondary base is formed from sphingosine by the loss of water. Also the structural formula for anhydrosphingosine given by Levene adds up to $C_{17}H_{33}NO$, and as far as can be made out from his paper, he considers his secondary product to be anhydrosphingosine.

Sphingosine

$$CH_3 . (CH_2)_{11}CH{=}CH . CH(OH) . CH(OH) . CH_2(NH_2).$$

Anhydrosphingosine

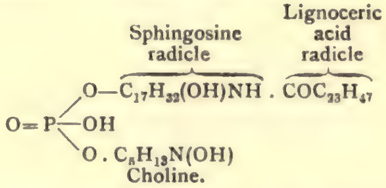
$$CH_3 . (CH_2)_{11}CH{=}CH . CH{=}C(OH) . CH_2(NH_2).$$

On reduction of this secondary base with hydrogen the resulting product possessed the structure of hydroxyheptadecylamine $C_{17}H_{34}(OH)NH_2$, and for this reduced base Levene proposed the name sphingine. Levene also made the important observation that the sphingosine was probably in combination with lignoceric acid in the sphingomyelin molecule, for he succeeded in isolating an intermediate product of hydrolysis which, after saturation with hydrogen in the presence of palladium, had the elementary composition of lignoceryl sphingine. This substance did not form salts with mineral acids; it gave no nitrogen on treatment with nitrous acid and therefore did not contain an (NH_2) group. It was probable therefore that the junction of lignoceric acid with sphingine took place through the (NH_2) group so that the compound might be represented by the formula

$$\underset{\substack{\text{Sphingine}\\\text{radicle.}}}{C_{17}H_{34}(OH)NH}{-}\underset{\substack{\text{Lignoceric}\\\text{acid}\\\text{radicle.}}}{CO . C_{23}H_{47}}$$

Only a small amount of this intermediate compound was obtained, and no further investigations were carried out on account of lack of material.

Since Levene's sphingomyelin yielded 34 per cent. of its weight in the form of sphingosine and 43 per cent. in the form of organic acids, and since the substance contained choline and phosphoric acid as well, it is obvious that there is no room in the molecule for the alcohol, sphingol, which Thudichum claimed to be present. Levene therefore believes that sphingomyelin does not contain sphingol. He suggests the structural expression for sphingomyelin :—



It will be seen that this formula, though differing in detail from that suggested by Thudichum, is based on the same general principle;

it allows only for one acid, and Levene explains the presence of the second acid, which he isolated on hydrolysis of sphingomyelin, by the assumption that the lignoceric acid may be substituted by this other acid. In this case sphingomyelin must be regarded as a mixture of two phosphatides differing only in their fatty acids. On the other hand, these two substances might be combined constituting a diphosphatide. These points are at present being investigated by Levene.

Elementary Analysis of Sphingomyelin.

Pure sphingomyelin has only been obtained recently [Levene, 1916], and all samples formerly investigated were contaminated with cerebrosides. The purest samples isolated before Levene succeeded in obtaining a pure specimen were described by Rosenheim and Tebb [1908, 1910, 1], but even these products contained some cerebroside. At present we have data for only four specimens of pure sphingomyelin, all isolated by Levene. The results of analysis of these substances are indicated in the following table, which also shows the percentage of sphingosine and acids obtained from sphingomyelin :—

Source.	C.	H.	N.	P.	Sphingosine, Per Cent.	Fatty Acids, Per Cent.	Ratio N : P.
Brain .	66·59	11·26	3·78	3·99	34·10	43·00	2·09 : 1
Kidney .	64·80	11·41	3·50	3·82	32·10	49·00	2·03 : 1
Liver .	64·47	11·57	3·41	3·81	32·14	41·70	1·98 : 1
Egg yolk	65·56	11·68	3·84	4·22	33·70	43·40	2·01 : 1

From the general agreement of these figures Levene conjectured that the sphingomyelins of brain, kidney, liver and egg yolk were identical. If sphingomyelin is a mixture of two phosphatides the slight difference in composition of the various specimens may be accounted for on the supposition that the proportion of the two substances varies somewhat in the material obtained from different organs.

Sphingomyelin-like Bodies.

Various substances having the properties of sphingomyelin have from time to time been isolated by different authors in the course of their work on phosphatides. Thus Stern and Thierfelder [1907] described a substance which they isolated from egg yolk and referred to as "Weisse Substanz". Thierfelder recognised that this body belonged to the sphingomyelin group of Thudichum. A similar substance was described by MacLean [1912, 2], who isolated it from horse kidneys. "Carnaubon," found in ox kidneys by Dunham and Jacobson [1910], and a corresponding substance isolated by MacLean [1913] from heart muscle, all come under this group; also the apomyelin and amidomyelin of Thudichum and the diaminomonophosphatide obtained by Fränkel and Offer [1910] from horse pancreas. All these substances proved to be diaminophosphatides (N : P = 2 : 1), but differed materially in elementary composition, and nearly all were found to yield a reducing sugar on hydrolysis. This reduction was due to the galactose present in the contaminating cerebroside, and we now know from the work of Rosenheim and of Levene that all these bodies were mixtures of sphingomyelin with cerebrosides. The extraordinary difficulty experienced in obtaining pure sphingomyelin is discussed later.

Comparison of the analytical figures obtained from these bodies with the figures obtained by Levene for pure specimens of sphingomyelin indicates that many of them must have contained considerable amounts of cerebrosides.

Observer.	Source of Material.	C.	H.	N.	P.	Ratio N : P.	Name Given to Substance by Investigator.
Rosenheim and Tebb [1908]	Brain	62·90	11·54	3·3	3·46	2·11 : 1	Sphingomyelin
Stern and Thierfelder [1907]	Egg yolk	68·15	12·14	2·77	3·22	1·9 : 1	"Weisse Substanz"
Dunham and Jacobson [1910]	Ox kidney	67·12	11·54	2·84	2·18	2·9 : 1	"Carnaubon"
MacLean [1912, 2]	Horse kidney	68·19	12·37	3·00	3·44	1·93 : 1	—
,, [1913]	Ox heart	—	—	3·07	3·41	1·99 : 1	—
Thudichum [1884]	Brain	67·01	11·35	3·00	3·23	2 : 1	Apomyelin
,, ,,	,,	65·37	11·29	2·96	3·24	2 : 1	Sphingomyelin
,, ,,	,,	62·4	—	—	—	—	Amidomyelin

It is a remarkable coincidence that mixtures of substances should give such close figures as the above show in certain cases. Even the determination of the so-called melting-point is not of much value in identifying these bodies, as the similarity of certain of their "melting-points," or more correctly their clearing-points (see p. 103), might suggest that they were definite chemical entities. Thus the substance from kidney "melted" at 182 to 183° and the heart substance at 184 to 185°, while a mixture of both "melted" at 183 to 184° [MacLean, 1913]. The most satisfactory test of purity is absence of carbohydrate material indicating absence of cerebrosides.

Chief Properties of Sphingomyelin.

The properties of sphingomyelin have been described by Thudichum [1884], Rosenheim and Tebb [1908; 1909, 1; 1910], Stern and Thierfelder [1907], and Levene [1913; 1914; 1916].

Sphingomyelin is a white non-hygroscopic stable substance which crystallises from alcohol in the form of tables or needles arranged in star-like formations. It can be easily pulverised to form a white powder, not unlike stearic acid in appearance but rather firmer and harder. On exposure to air and light, sphingomyelin undergoes no change. It dissolves to a very slight extent in cold alcohol but very easily on heating; from its hot alcohol solution sphingomyelin separates on cooling in crystalline form. In cold or hot ether it is practically insoluble, but dissolves easily on warming in chloroform, benzene, pyridine and glacial acetic acid. In cold acetone it is insoluble, but dissolves to a certain extent in hot acetone.

With water, sphingomyelin forms an opalescent starch-like mixture from which it is precipitated by acetone. It forms combinations with cadmium chloride, platinum chloride and lead acetate. When quite pure it gives no carbohydrate reaction. On treatment with nitrous acid by Van Slyke's method no nitrogen is evolved, indicating the absence of free amino nitrogen in the molecule. Dissolved in glacial acetic acid and alcohol, sphingomyelin absorbs hydrogen. For an impure specimen the iodine value was found to be 34·3 [Stern and Thierfelder]. Sphingomyelin is dextro-rotatory, material from different sources having about the same specific rotation as shown by the following figures given by Levene [1916]:—

Source of Sphingomyelin.	Solvent.	Concentration.	Temperature.	$[\alpha]^{D}$ Value.
Brain . .	Chloroform and methyl alcohol 1 : 1	about 8 per cent.	25°	+ 8·20
,, . .	,, ,, ,,	,, ,,	,,	+ 8·20
,, . .	,, ,, ,,	,, ,,	,,	+ 7·53
,, . .	,, ,, ,,	,, ,,	,,	+ 7·52
Kidney .	,, ,, ,,	,, ,,	36°	+ 8·73
Liver . .	,, ,, ,,	,, ,,	32°	+ 7·61
Egg yolk .	,, ,, ,,	,, ,,	25°	+ 7·54

CHAPTER III.

OCCURRENCE, METHODS OF EXTRACTION, ISOLATION AND PURIFICATION OF PHOSPHATIDES.

Occurrence of Phosphatides in the Tissues.

HOPPE SEYLER [1867] made the observation that when egg yolk was treated with ether until no more extract could be obtained, the egg-yolk residue still contained phosphatides which could be removed by treatment with warm alcohol. Subsequent investigators [Erlandsen, 1907; Cohn, 1911] have confirmed this observation, and it is now generally recognised [Schulze and Steiger, 1889] that the same phenomenon is met with in all tissues, both animal and plant. Thus in extracting dried healthy kidneys with ether not more than 15 per cent. to 30 per cent. of the total fats and lipins, and often less than this, can be obtained. On subsequent treatment with alcohol the remainder comes out with ease. Heart, liver, pancreas, muscle, blood, milk and various body fluids, etc. all behave in the same way. No matter how long the ether extraction is persevered with, a large percentage of the phosphatides still remains in the tissue.

Attempts have been made to explain this phenomenon on the supposition that the ether fails to penetrate the tissues—that the difficulty is simply a mechanical one. It has been pointed out that when fats or phosphatides are mixed with protein matter and the whole mass dried and then extracted with ether, only a fraction of the added ether-soluble material is removed. While this is true to a degree, it is equally certain that the fraction which can be removed bears a much higher proportion to the total fatty matter than is the case in extraction of the tissues.

Again, if the organ extracted is not a healthy specimen but one suffering from marked fatty degeneration, the greater part of the phosphatides is obtained by ether extraction.

From this it would appear that part at any rate of the phosphatide material is present in some kind of ether-insoluble combination with the proteins; this combination is acted on by alcohol and the phosphatide liberated; it is then easily extracted by ether in so far as physical conditions permit. In this connexion an interesting observation was made by Osborne and Wakeman [1915]. In an investigation on the lecithin of milk they found that the phosphatides were present in the precipitated protein matter. On drying this protein and extracting it with ether, no lecithin whatever was obtained, though subsequent treatment with alcohol removed a considerable amount.

The exact nature of this association-complex between lecithin and protein is not known. Hoppe Seyler [1867] advanced evidence in support of the view that part of the lecithin of egg yolk was in direct chemical combination with the protein. Others believe that the association is dependent on physical phenomena [Fischer and Hooker, 1916], and the latter view is now generally held.

Judging from experimental results it would appear that much of the *essential* fatty matter of cells is in lipin form and is chiefly composed of lecithin and kephalin in intimate association with the cell protein. This essential "fat" appears to be necessary for the survival of the cell, since it is preserved under circumstances of extreme emaciation [Mayer and Schaeffer, 1913]. Whether it represents a stage in the elaboration of fat to more complicated compounds is a point to be considered, but of this we know nothing with certainty [Hammersten, 1904; MacLean and Williams, 1909]. According to Heffter [1891, 1] lecithin probably forms fat under certain conditions; that it plays a most important part in the processes of vital activity is suggested by its universal presence in all living cells.

The combination of phosphatides with cell protein explains the difficulty encountered in attempting to stain many normal tissues for fat. In the case of the normal kidney, for instance, no fatty material can be demonstrated by any of the ordinary staining methods, but if the kidney is one showing signs of fatty degeneration, then fat can be easily demonstrated and stains well. In the latter case the lipins which are naturally in combination are in the free state and give the ordinary staining reactions of fats, while under normal circumstances the combined form is present and gives no reaction with fatty stains.[1] In true "fatty infiltration" the ether extract of the tissue contains large amounts of neutral fats and fatty acids but only the normal amount of lipins; in "fatty degeneration," on the other hand, the combined lipins are set free so that nearly all the fatty material is removed by ether extraction. The serious significance of "fatty degeneration" as against "fatty infiltration" is thus obvious, seeing that the former condition implies the disintegration of the cell tissue, whereas infiltration generally indicates some difficulty on the part of the cell to deal with reserve fat. No doubt, in many cases, the primary defect in cellular activity is progressive and leads to true degeneration, but fatty infiltration of itself is not necessarily associated with lessened activity; on the other hand, fatty degeneration is inconsistent with the continuance of the functional activity of the cell.

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[1] See also Fischer and Hooker (1916).

Amount and Estimation of Phosphatides in the Tissues.

Various attempts to estimate the lecithin (phosphatide) content of tissues have been made. From the nature of the problem, however, we are still without any reliable information on this point, since no method giving satisfactory results has as yet been evolved. Attempts to isolate and weigh the phosphatide as such are attended with much loss so that the figures are generally too low. By this method Erlandsen [1907] obtained from dried ox heart from 7 to 10 per cent. of lecithin, from egg yolk Roaf and Edie [1905] obtained 9·5 per cent., while Manasse [1906] obtained a similar figure. It is probable that the proportion of lecithin present in egg yolk varies very considerably and is dependent on various factors [MacLean, 1909, 1; Tornani, 1909].

For various tissues hardened in formaldehyde Cruickshank [1913, 1] gives the following numbers for 100 grms. of wet substance:—

Ox heart	0·36 grm.
„ kidney	0·48 „
„ spleen	0·14 „
„ lungs	0·40 „
„ testicles	0·62 „
„ thyroid	0·30 „
„ pancreas	0·68 „
„ submaxillary	0·30 „
„ red corpuscles	3·50 grms.
Sheep red corpuscles	0·12 grm.
Human brain	0·60 „

The majority of the results obtained in attempts to estimate the phosphatide content of tissues have been arrived at by extracting the tissue with suitable organic solvents and estimating the phosphorus content of the more or less purified extract. From this the amount of "lecithin" is reckoned on the assumption that lecithin contains about 4 per cent. of phosphorus.

Unfortunately, there are various indications that phosphorus-containing bodies other than lipins are extracted along with the phosphatides, so that at best the method gives only very rough indications. Probably the suggestion of Collison [1912] that the solvents used should be water-free is of value, but even then it is difficult to get the tissues absolutely water-free. A general idea of the figures so obtained is furnished by the following table quoted from Nerking [1908]. An attempt to estimate separately the amount of lecithin and kephalin in the tissues was made by Koch and Woods [1905], but almost all investigators have simply extracted the mixture of phosphatides present and called the extract "lecithin".

	Percentage of Lecithin in "Wet" and Dried Tissues of *Cat.*		Percentage of Lecithin in "Wet" and Dried Tissues of *Rabbit.*		Percentage of Lecithin in "Wet" and Dried Tissues of *Hedgehog.*	
	"Wet" Tissues.	Dried Tissues.	"Wet" Tissues.	Dried Tissues.	"Wet" Tissues.	Dried Tissues.
Lung . . .	1·36	6·10	1·52	5·96	0·85	4·28
Heart . . .	1·27	4·55	1·60	5·86	2·09	10·49
Brain . . .	4·48	13·74	3·86	12·41	4·18	22·31
Cord . . .	9·52	26·20	11·16	35·18	6·47	18·19
Kidney . . .	1·69	6·26	1·34	5·02	1·88	8·55
Spleen . . .	0·09	0·39	1·19	4·23	1·44	6·56
Eye . . .	—	—	0·35	2·19	—	—
Liver . . .	1·39	4·99	1·07	3·82	1·45	5·23
Stomach . .	—	—	0·88	3·31	1·11	6·37
Intestines . .	—	—	0·21	0·62	0·24	1·50
Blood . . .	—	—	0·14	0·86	—	—
Muscle . . .	—	—	0·60	2·59	1·00	3·71
Bone marrow .	—	—	2·71	—	41·7	—
Bone . . .	—	—	0·18	0·27	0·59	0·87
Skin . . .	—	—	0·20	0·48	0·35	0·58
Suprarenals . .	1·98	5·36	2·39	5·54	21·23	92
Testicles . .	—	—	1·03	3·39	1·92	11·27

Various other estimations for different organs have been carried out, but since the results are so variable no purpose is served in giving figures. Among these may be mentioned investigations on brain and spinal cord [Fränkel, 1909; Fränkel and Linnert, 1910, 2; Fränkel and Dimitz, 1910], lung [Sieber, 1909], bone marrow [Glikin, 1907; Otolski, 1907; Bolle, 1910], heart muscle [Rosenbloom, 1913, 1], suprarenals [Mulon, 1903; Bernard, Bigart and Labbé, 1903], placenta [Bienenfeld, 1912], fæces [Long, 1906], blood [Hermann and Neumann, 1912; Grimbert and Laudat, 1912; Bürger and Beumer, 1913; Dinkin and Klein, 1914; Bloor, 1915; 1915, 1; 1916; Bloor, Joslin and Hornor, 1916], and various other tissues [Gerard and Verhaeghe, 1911; Virchow, 1911; Cohn, 1913].

The question of the lecithin content of milk has naturally engaged considerable attention. Milk as food for the young might be expected to contain much phosphatide, but this is not the case. In fact, more than one observer has argued that it contained no phosphatide whatever, but it is now certain that a small amount is present. Nerking and Haensel [1908] found 0·024 to 0·079 per cent. in human milk, and ·062 to ·116 per cent. in cow's milk. Other observations on milk phosphatides are by Tolmatscheff [1867], Stoklasa [1897], Burow [1900], Bordas and Raczkowski [1902], Koch and Woods [1905], Koch [1906], Glikin [1909], Brodrick-Pittard [1914] and Osborne and Wakeman [1915]. An attempt by Wrampelmeyer [1893] to distinguish between margarine and butter by estimating the lecithin content of the latter was unsuccessful.

Another important fluid—blood—has given various results, the most exact probably being those of Bloor [1915] who found that dog's blood had a lecithin content of 0·36 to 0·42 per cent. and human plasma 0·21 to 0·28 per cent. Marked differences between the lecithin content of the blood of certain animals is referred to by Nerking [1908]. According to Bürger and Beumer [1913] very little lecithin is present in the blood of man and the sheep; sphingomyelin is most abundant and then kephalin and a so-called water-soluble phosphatide.

Methods of Extraction, Isolation and Purification of Phosphatides.

Many of the principles governing the extraction of phosphatides from the tissues will be obvious from the above remarks. It is clear that extraction by ether alone is unsuitable, since only a small amount of the phosphatide material is dissolved. Practically all the methods, both old and new, depend on the employment of ether and alcohol. Riedel [1912] recommended the use of methyl alcohol. The more important of the older methods will be referred to, and after the general principles have been explained, the individual phosphatides —lecithin, kephalin, sphingomyelin and cuorin—will be treated in detail.

Older Methods of Obtaining Phosphatides (Lecithin).

In the earlier attempts to obtain lecithin[1] great difficulty was experienced in the separation of this substance from fats and cholesterol owing to the common solubility of all these substances. This difficulty was to a great extent overcome by taking advantage of Hoppe Seyler's observation already described. Hoppe Seyler [1867] and Parke [1867] thoroughly extracted egg yolk with ether and treated the residue with warm alcohol. The alcohol extract obtained was concentrated and on cooling gave a precipitate of a substance containing nitrogen and phosphorus which Hoppe Seyler considered to be lecithin. The latter observer states that this lecithin was obtained by him in crystalline form as fine glistening needles, but subsequent observers have failed to secure this result. From the method of preparation it is quite certain that this lecithin must have contained an excess of kephalin; the latter substance is much less soluble in alcohol than lecithin and would thus tend to fall out on cooling. It is therefore difficult to understand how crystals of phosphatide could have been obtained, and it seems more probable that Hoppe Seyler's lecithin crystals were crystals of fat or fatty acids which contaminated the lecithin.

This method of obtaining lecithin was employed by Diacanow [1868, 3] whose work on the chemical constitution of lecithin was carried out on a substance isolated according to Hoppe Seyler's principles. Diacanow used egg yolk, and after extracting with ether, cooled the subsequent alcohol extract to – 10° or so. He thus obtained what he considered to be pure lecithin. A similar method was used in the preparation of lecithin from brain [Diacanow, 1868, 1].

Strecker [1868], who also carried out an important investigation on the structure of lecithin, obtained his substance in a different way. Strecker added platinum chloride to the alcohol-ether extract of egg yolk and precipitated the phosphatide as the platinum combination. This compound was dissolved in ether, precipitated by alcohol several times, and the lecithin separated from the purified product by hydrogen sulphide.

Gilson [1888] prepared lecithin from the *ethereal* extract of egg yolk by evaporating off the ether, dissolving the residue in petroleum ether and washing the petroleum ether solution in a separating funnel with several changes of 75 per cent. alcohol. On allowing the alcohol solutions to stand in a cool place, a precipitate settled out which was filtered off. The solution was then decolorised by means of animal charcoal, the alcohol evaporated and the residue taken up with ether. The ethereal solution was filtered, evaporated to dryness, and the residue taken up in a very small amount of alcohol. On evaporation at low temperature lecithin was obtained.

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[1] The only phosphatide considered was lecithin.

Thudichum [1884] obtained lecithin from brain by a very complicated method. The brain was extracted with warm alcohol, and the alcohol extract, after undergoing various processes, treated with cadmium chloride. The cadmium chloride compound was thoroughly exhausted with ether and the ether-insoluble residue treated with cold benzene. The part soluble in cold benzene Thudichum recognised as lecithin cadmium chloride, and from this "lecithin hydrochlorate" was separated by sulphuretted hydrogen. This "lecithin hydrochlorate" was separated as a felted mass of crystals. This sample must have been "true lecithin" with the whole of its nitrogen present in the form of choline, since extensive preliminary precautions were taken to get rid of kephalin; any kephalin precipitated by the cadmium chloride would have been dissolved out by the treatment of the cadmium compound with ether. Indeed Thudichum mentions that "the quantity of the platinum salt obtained was very near to that which should have been obtained if all the nitrogen had existed in one form in lecithin and had been obtained in one form".

All these methods were unsatisfactory, and many of them exceedingly tedious as well. Also the manipulation necessary in some of them could not have failed to produce changes in such a labile substance as lecithin. The isolation of phosphatides in a pure form was rendered much simpler by the important discovery of Zuelzer [1899] that phosphatides were insoluble in acetone.[1] By this medium the ether or alcohol extract of a tissue can be easily separated from ordinary fats, fatty acids and cholesterol. In all investigations subsequent to Zuelzer's observation acetone has been used in the separation of phosphatides. Of the older methods the only important remaining one is that of Bergell.

Bergell [1900] extracted egg yolk for six hours with boiling 96 per cent. alcohol under a reflux condenser. After cooling to 0°, an alcoholic solution of cadmium chloride was added, the mixture allowed to stand for some hours, the precipitate separated and washed with 96 per cent. alcohol. The precipitate was then dried in air, extracted with ether, and boiled under a reflux condenser with eight times its volume of 80 per cent. alcohol. Ammonium carbonate in concentrated solution was gradually added to the boiling mixture until the fluid was alkaline, and a test portion showed the filtrate to be cadmium free. During the addition of ammonium carbonate the flask was thoroughly shaken. On completion of the reaction, the solution was filtered hot and the filtrate gradually cooled to – 10°. The resulting precipitate was taken up in chloroform, precipitated with acetone and dried *in vacuo* over sulphuric acid.

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[1] Probably this fact was known before Zuelzer's observation, but he was the first to adopt it for practical purposes.

This method has been used with certain modifications by Schulze and Winterstein [1903], Hundeshagen [1883], Lüdecke [1905], MacLean [1915] and others, but is objected to by certain investigators on the ground that the lecithin is decomposed on treatment with cadmium chloride with the liberation of part of the fatty acids [Erlandsen, 1907; Eppler, 1913]. Since the substances used by Erlandsen and others were impure, or at any rate consisted of mixtures of lecithin and kephalin, it is doubtful whether this objection is valid. The N : P ratio of Bergell's lecithin was exactly 1 : 1, the nitrogen being 1·74 per cent. and the phosphorus 3·75 per cent. As this is about the average figure for lecithin, it would not appear that much decomposition had taken place [Thierfelder, 1909].

Recent Methods for Obtaining Phosphatides.

In all the later methods for obtaining phosphatides the tissue is first freed from macroscopic fat and then dried. Owing to the labile nature of certain of the phosphatides and the ease with which oxidation of their unsaturated fatty acids takes place, the problem of drying is not so simple as at first sight it might appear. The easiest way to get rid of water is to expose the pulped tissue to a high temperature in an oven or water-bath, and this method has been actually adopted by certain investigators. Such methods, however, are quite inadmissible in dealing with phosphatides. The objects to be aimed at are to get the tissue dried in the shortest possible time with the minimum of exposure to air and at the lowest temperature that can be conveniently employed. Only in this way can one hope to isolate phosphatides in an unchanged condition.

Methods of Drying Tissues.

Drying in Air.

Erlandsen [1907] dried minced heart tissue by spreading out the finely minced material in a thin film on a glass plate. The temperature was raised to about 30° by means of Bunsen burners, and a current of air generated by an ordinary electric fan made to play over the material. When dry, the substance was broken into small fragments and passed through a grinding machine; the process of drying was completed by putting the powder obtained into an evacuated desiccator containing sulphuric acid.

This is a very efficient method for dealing with tissues or egg yolk, and quite a considerable amount of material can be dried in a comparatively short time. The process is greatly accelerated if the material is broken up at intervals, since a skin tends to form on the surface which prevents further drying. For fear of setting up decomposition the temperature should not exceed 30°. In cases where the substance to be dried contains a great deal of fat, it may be necessary to treat it with alcohol or ether after drying and prior to reducing it to powder. This method has been used successfully by Stern and Thierfelder for egg yolk [1907], by MacLean for egg yolk, heart and kidney tissue [1909, 1912, 2] and by others. The chief objection to its use appears to be the possibility of autolytic processes taking place. The conditions are suited both for autolysis and for the development of bacteria, and it is uncertain whether some decomposition and oxidation may not occur in certain cases. Probably in investigations which require quite unchanged substances one of the following methods is preferable. The plan suggested by Cruickshank [1913], in which the tissues are fixed with formaldehyde previous to extraction, is likely to produce changes in the lipins and possesses no obvious advantages.

Drying by Alcohol.

The tissue after being thoroughly pulped is covered with several times its volume of alcohol. The mixture is well stirred, allowed to stand for a short time and the liquid separated by filtration through a cloth. The residue thus obtained is packed up in sail cloth and subjected to pressure by means of a laboratory hand press; by this means almost all the alcohol is squeezed out. A firm cake remains, which after drying at room temperature under the fan for half an hour or so can be ground into powder and extracted with ether or other solvent. The alcohol used for drying is diluted considerably by the water of the tissues, and this aqueous alcohol does not remove much phosphatide.

Drying by Acetone.

This is perhaps the best method of all, and has been used by Fränkel, Parnas and others. Theoretically, acetone has an advantage over alcohol in that the former solvent dissolves fats and cholesterol, but leaves the phosphatides behind. Practically, it is found that acetone is capable of removing some phosphatide, for lecithin is apparently soluble to a small extent in acetone which contains fatty substances in solution [Erlandsen, 1907; MacLean, 1914, 1]. Under the conditions generally employed the amount removed is very small, but if boiling acetone is used, as was the case in Fränkel's work, considerable amounts of phosphatides are dissolved. By the use of acetone at ordinary temperature a tissue may be freed from water, and at the same time fats and cholesterol dissolved out, the only fatty bodies left behind being the lipins. Acetone is used in the same way as alcohol.

Drying by Anhydrous Salts.

When certain anhydrous salts are rubbed up intimately with moist tissue, water is taken up and the hydrated salt formed. In this way a dry powder can be obtained which is suitable for extraction by the usual solvents. Anhydrous sodium sulphate was employed for this purpose by Pinkus [1901] and Schryver [1906]; Rosenheim [1906] used the calcium salt. According to Fränkel and Elfer [1910; 1912] anhydrous sodium phosphate is preferable both to sodium and calcium sulphates. This method has the disadvantage that large amounts of the anhydrous salts are required to give a dry mixture, but is suitable for drying small amounts of tissue; when large amounts have to be dealt with, as is generally the case in phosphatide investigations, the dried mass obtained is so voluminous as to render the method impracticable. Calcium carbide has been recommended by Rosenbloom [1913], but for obvious reasons it can hardly be recommended.

Extraction and Isolation of Phosphatides from Dried Material.

The material which has been dried by one of the above processes is now thoroughly extracted with a suitable solvent. For this purpose ether and alcohol are the solvents generally employed. Sometimes the dried material is first exhausted with ether and the residue subsequently extracted with alcohol. This method was used by Erlandsen [1907] and considered by him to be necessary, since this investigator found that the phosphatides removed by the ether were different from those found in the subsequent alcohol extract. This observation of Erlandsen was found to be erroneous, and MacLean [1913] has shown that the phosphatides in the alcohol extract are the same as those of the ether extract. Thus in cases where it is convenient to employ alcohol alone as a solvent there are no objections to its use.

Whatever plan is adopted, the resulting extract contains not only phosphatides but fat, fatty acids, cholesterol and other substances as well.

In an ether extract the phosphatides which may be present are lecithin, kephalin, sphingomyelin and cuorin, whereas an alcohol extract contains the first three substances but no cuorin.

Since lecithin is easily soluble in both alcohol and ether its presence in both extracts is easily understood. Kephalin, though soluble in ether, is generally regarded as being insoluble in alcohol, but this is only relatively true as already pointed out. Possibly also the presence of other fatty matters renders it still more soluble in this solvent; at any rate the alcohol extract of a tissue invariably contains a certain amount of kephalin.

Sphingomyelin, though practically insoluble in pure ether, is fairly soluble in an ethereal extract of lecithin; it is somewhat soluble in cold alcohol and easily soluble in warm alcohol. Its presence in both ether and alcohol extracts is thus explained.

Cuorin, regarding the occurrence of which some doubt exists, is apparently quite insoluble in alcohol; it is therefore not found in alcoholic extracts.

General Principles of Separation of Phosphatides from Associated Substances.

After thorough extraction of the tissue, the extract is concentrated and the residue treated with ether when a thick opalescent mixture is obtained. The insoluble fraction consists partly of impurities, such as inorganic salts and a nitrogenous substance to be described later, and partly of sphingomyelin and cerebrosides. This ether-insoluble part is separated either by filtration or by centrifuging, leaving a clear yellowish ether solution. This solution is concentrated and treated with excess of acetone, which precipitates the phosphatides leaving cholesterol and fats in solution. On redissolving the precipitate in ether, a slightly opalescent solution is again obtained; the opalescence is due to the presence of small amounts of a mixture of sphingomyelin and cerebrosides. On centrifuging, the liquid yields a clear solution which is concentrated and precipitated with acetone as before. The precipitated phosphatides are now comparatively free from cholesterol, fat, fatty acids and sphingomyelin; by several repetitions of the above process of dissolving in ether and precipitating with acetone, every trace of these impurities is ultimately got rid of. The phosphatide mixture isolated in this way is dissolved in a small amount of ether and excess of absolute alcohol added. The addition of alcohol causes some of the phosphatide to separate; this alcohol-insoluble part constitutes crude kephalin and may contain some cuorin. The alcohol-soluble part when further purified yields a substance with a nitrogen and phosphorus percentage corresponding to that of lecithin and is considered to be pure lecithin.

Many modifications of the above processes may be introduced to suit particular cases,[1] but the essential manipulations are always the same. The isolation of the individual phosphatides in a pure state is described later. Some observations by Erlandsen already referred to regarding the nature of the phosphatide present in the alcohol extract of heart muscle require consideration at this later stage.

Erlandsen's Investigation on Heart Muscle Phosphatides.

Erlandsen [1907] extracted dried heart tissue with several changes of ether till little or no residue remained on evaporating the ether. On further extraction with alcohol, a phosphatide was obtained which was not lecithin. This substance was isolated as a cadmium chloride combination and was found to be a diamino-monophosphatide. The ethereal extract on the other hand contained lecithin, as was to be expected, together with an alcohol-insoluble phosphatide which had all the properties of kephalin. On analysis it was found, contrary to expectation, that this body differed in composition from kephalin since its N : P ratio was not 1 : 1 as in kephalin, but 1 : 2. To this substance Erlandsen gave the name cuorin.

In the course of an investigation on horse kidney, MacLean [1912, 2] found that the phosphatides of the primary ether extract were of the same nature as those described by Erlandsen for heart muscle, but that the phosphatide of the alcohol extract contained very variable amounts of nitrogen and gave N : P ratios varying from 4 : 1 to 1·5 : 1. This suggested that the phosphatide present was contaminated by a substance or substances containing a high percentage of nitrogen, and that some light might be thrown on the nature of this phosphatide if it could be separated from the nitrogenous substance. This impurity gave almost all the ordinary reactions of phosphatides, so that all earlier attempts failed to effect complete separation though material changes in the N : P ratio were in evidence. Finally it was found that repeated emulsification of the alcohol phosphatide mixture with water, followed by the addition of a small amount of acetone, resulted in a purification of the phosphatide, and on analysis the N : P ratio proved to be the same as that of lecithin—1 : 1. The purified substance gave all the ordinary lecithin reactions. There was therefore no doubt that the phosphatide present in horse kidneys, which was extracted by alcohol after preliminary treatment with ether was of the same nature as the lecithin obtained in the preliminary ether extract—a monoamino-monophosphatide. Similar experiments carried out on heart muscle gave identical results, indicating that Erlandsen's diamino-monophosphatide was simply impure lecithin [MacLean, 1913]. The nitrogenous impurity, which is always present in alcohol extracts of organs, is satisfactorily separated only by the method described. The substance is responsible for certain of the alleged new phosphatides, many of which are nothing more than lecithin contaminated with this product. The nature of this contaminating substance is unknown and it is no doubt a mixture, though from its general properties a good deal of some body representing a chemical unit must be present. How far it is formed as the result of decomposition processes in the tissue is unknown, but this factor probably plays a part. As it will be necessary to refer to this substance on several occasions some term to designate it is almost essential. For purposes of reference the writer will in future pages refer to this nitrogenous impurity as "Carnithin"—a name which is provisionally suggested merely for reasons of expediency and with no relation to its chemical composition. Some properties of this body are given below.

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[1] See for instance Fränkel [1909, 1], who describes a method of extraction of lipins from the brain.

Carnithin.

Carnithin is obtained from the phosphatide fraction present in the alcohol extract of the tissues. On emulsifying the impure phosphatide mass with water and then adding acetone, the phosphatides separate leaving behind a slightly brownish fluid. On evaporating this fluid, carnithin is left behind as a soft sticky syrupy residue.

Carnithin is insoluble in ether and in *absolute* alcohol, but easily soluble in alcohol containing traces of water. It is exceedingly soluble in water; on standing for some days an aqueous solution gradually deposits small round white crystals. These crystals are insoluble in cold water, but dissolve in boiling water and are reprecipitated on cooling. After recrystallisation in this way several times, a substance or mixture of substances is obtained which has a very high nitrogen content and appears to belong to the purine class of bodies. In one case small round concentric white crystals having all the properties ascribed to carnine were isolated. After drying at 105° this crystalline substance was found to contain 28·55 per cent. nitrogen. On heating it changed colour at about 230° and blackened at 240°. With silver nitrate a white flocculent precipitate was obtained which did not dissolve in ammonia or nitric acid. A precipitate was also given with basic lead acetate and with mercuric chloride, but not with lead acetate nor with mercuric nitrate. In another sample of carnithin a small amount of a substance which appeared to be impure hypoxanthine was isolated; it contained about 40 per cent. of nitrogen. The mother liquor from which these substances separate is distinctly acid in reaction, and it is probable that these bodies are set free by a process of decomposition and are not present in the free state in the original liquid. This is suggested by their extreme insolubility in cold water, and the difficulty experienced in redissolving them in the mother liquor after separation has once taken place. The fact that complete separation takes several days or even weeks is also suggestive in this connexion.

Owing to the high nitrogen percentage in these bodies, it is obvious that the presence of a small quantity of this impurity in lecithin would materially raise the nitrogen percentage and considerably modify the N : P ratio. When the separation of these products is complete, an absolutely clear straw-coloured mother liquor is obtained which remains clear for a very long time. On evaporating to dryness, a sticky hard gumlike substance remains. This body which dissolves in alcohol containing some moisture is also soluble in solutions of lecithin. *Further, like phosphatides in general, it is precipitated by acetone, by cadmium chloride and also by platinum chloride.* Its cadmium chloride salt is soluble in warm benzene, but is precipitated from this solution by absolute alcohol. The substance appears to be of a very complex nature, and so far no clue as to its chemical composition has been obtained. Different specimens were found to contain on an average about 6 per cent. of nitrogen, and there is generally a small amount of phosphorus also present, though the latter may not amount to more than a trace and may even be absent. When tested by Van Slyke's method a considerable part of its nitrogen was evolved. On hydrolysis some specimens yielded a small amount of fatty acids, but this was probably due to contamination with lecithin, since a specimen has been obtained which when boiled with hydrochloric acid for several hours gave no trace of fatty acids. It decolorised Fehling's solution on heating but no precipitate occurred; on prolonged boiling, however, a dense precipitate was formed. Attempts to fractionate this body showed that it contained a substance which gave the reactions of creatinine.

Carnithin, though quite insoluble in pure ether, is easily soluble in an ethereal solution of lecithin, so that this body cannot be separated from lecithin by ether extraction; the same holds good for alcohol. From descriptions of the methods employed by several investigators, it is quite obvious that many of the supposedly pure phosphatides isolated must have been contaminated with this impurity.

Detailed Methods for Isolation and Purification of the Individual Phosphatides.

The general principles on which the isolation of phosphatides is based are sufficiently indicated above. Various modifications and extensions may be introduced to suit particular circumstances, and there is no single method which can be claimed to give the best results in all cases. Details of one method for the isolation of each of the principal phosphatides are given below, as it was thought that this plan might be helpful to anyone attempting to prepare phosphatides for the first time and having no practical experience of these bodies. It is obvious, however, that all the phosphatides may be isolated at the same time from the same tissue.

I.

Preparation of Lecithin [MacLean, 1914].[1]

In the preparation of lecithin the most suitable solvent to use is alcohol, though of course ether may also be employed. The various steps in the process are as follows:—

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[1] The method described here is a slight modification of that published in the "Journal of Pathology and Bacteriology," **18**, p. 490.

1. The tissue used (heart, kidney, egg yolk, etc.) is, in the case of solid organs, ground to a fine paste by a mincing machine and dried by one of the methods described. On the whole, it is perhaps preferable to dry the tissue by means of acetone, but alcohol or the air process may be used. Whatever method is employed, the resulting product is passed through a coffee mill and ground to a fine powder.

2. The dried powder is thoroughly extracted several times (generally four to six) with excess of absolute alcohol. The best way to do this is to use large well-stoppered bottles, and to shake the mixture of alcohol and tissue by means of a shaking machine. After shaking for two to three hours the bottle is removed, and left to stand for some time when the bulk of the alcohol can be decanted off. It is not necessary that the separated alcohol should be quite clear. Sometimes the bottle containing the alcohol and tissue is filled with carbon dioxide to prevent oxidation as far as possible. If any difficulty is experienced in separating the alcohol, the mixture may be filtered either through a cloth or filter paper. The latter process is rather slow and is seldom necessary; if, however, it should be used filtration may be facilitated by the use of the suction pump. In ordinary cases all that is necessary is to decant as much alcohol as possible and pass the remainder through a suitable cloth. This of course gives an opalescent fluid, but there does not seem to be any special advantage in getting a clear filtrate at this stage. The various extracts are mixed and preserved in well-stoppered bottles. On completion of extraction the mixed alcoholic extracts are concentrated to small bulk under reduced pressure at 40°.

3. The residue is taken up with a small volume of ether in which much of it remains insoluble. To the mixture, without any attempt at filtration, acetone is added in excess and the resulting precipitate pounded together by a pestle and separated. The precipitate is again mixed with ether, precipitated by acetone and treated as before. By this process, which is repeated three or four times, all but traces of acetone-soluble bodies are removed.

4. The precipitate so obtained is rubbed up in a mortar with a large excess of water and a good emulsion made; to the emulsion from one quarter to one half its volume of acetone is added. On the addition of the acetone a large amount of substance separates in the form of large white flakes which float on the surface of the liquid. This is removed, partly by means of a glass spatula and partly by filtration through a filter paper perforated by small holes. It is again

emulsified and precipitated three times more. In order to get the phosphatides to separate well it is advisable to add a little sodium chloride to the aqueous emulsion prior to the addition of the acetone; indeed the *emulsification* can be carried out with water containing a little sodium chloride, and in this case the phosphatides always separate very easily.

The filtrate obtained from the first emulsification always contains much soluble matter—inorganic salts and the nitrogenous impurity already referred to (Carnithin)—but after the first treatment only traces of carnithin are found in the liquid.

The solid substance which separates is now dried by treating it several times with fresh additions of acetone. Finally as much acetone as possible is pressed out of the mass by means of a pestle, and the whole taken up in ether in which it is still partly insoluble forming an opalescent mixture.

5. The ether mixture is centrifuged when a clear supernatant fluid and a white precipitate are obtained. This white precipitate is impure sphingomyelin. The ethereal solution is decanted off and treated with excess of acetone. The resulting precipitate is again taken up in ether when an almost clear solution should be obtained. Centrifuging is repeated as before, and the clear supernatant ethereal solution again precipitated with acetone. Generally it is only necessary to centrifuge twice, but if the phosphatide is not quite soluble in ether after a second treatment the process must be repeated until a precipitate is obtained which is quite soluble in this solvent. If a centrifuge is not available separation may be accomplished by allowing the mixture to stand for some time in narrow tall cylinders; the solid part gradually falls to the bottom and the ether can be decanted off. Ordinary filtration is not very successful in these cases.

6. The substance is now dissolved in alcohol, and if the solution is not quite clear it is allowed to stand for some time until the insoluble substance (crude kephalin) settles on the bottom of the flask. The amount of alcohol-soluble substance obtained depends on circumstances to be explained later. After separation of the bulk of the alcohol-insoluble part by decantation, the solution is filtered.

7. The clear alcohol solution is now concentrated under reduced pressure at 40° until all the alcohol is removed. The residue is taken up in ether and the phosphatide separated from the ethereal solution by acetone. The precipitate obtained should give a perfectly clear solution with ether and with alcohol. It is finally treated with several

changes of acetone and dried in a desiccator *in vacuo* over sulphuric acid. This substance corresponds to "lecithin" and should show on analysis about 1·8 per cent. of nitrogen and 4 per cent. of phosphorus giving a nitrogen : phosphorus ratio of approximately 1 : 1.

The lecithin isolated in this way should be quite free from impurities, but as already explained is not "true lecithin" but a mixture of true lecithin and kephalin. It possesses, however, all the properties of the substance generally alluded to as lecithin. This lecithin can if desired be separated into its components by the method already described.

Some Anomalous Results Obtained in the Preparation of Lecithin.

When a tissue is treated as above described a good deal of alcohol-insoluble phosphatide (kephalin) is usually obtained (see paragraph 6). If, however, the same kind of tissue is procured in *as fresh a condition as possible and quickly dried*, it is often found that the phosphatide ultimately obtained is entirely soluble in a small amount of alcohol. In this case the whole of the extracted phosphatide, since it is soluble in alcohol, would be held to be lecithin. Now these divergent results can be obtained from the same tissue, so that according to the treatment of the material, one observer may find nothing but lecithin while another may find abundance of both lecithin and kephalin. Again it very often happens that lecithin which dissolves easily in alcohol immediately after its separation from the tissues becomes partly insoluble in alcohol, even when preserved in a carefully evacuated brown glass desiccator. The alcohol-insoluble substance formed has the properties and composition of kephalin. This change in solubility is much more in evidence if the organic solvents employed are not quite pure, and in general the greater care taken to purify the various organic reagents (ether, acetone, etc.) the more lecithin is obtained. Particularly does this appear to be the case with ether. This action of ether has been observed by Thudichum [1884] and by Fränkel [1909, 1]. These observers found that this solvent, unless specially purified, produced marked changes, and tended to render lecithin insoluble in alcohol. Parnas [1909], however, denies that ether possesses any deleterious effect, but as he was working with kephalin (which is itself insoluble in alcohol) it is difficult to see how he could have found any change. There is no doubt whatever that the ether supplied in England, even the best grades, has a marked effect on the alcohol-soluble lecithin-kephalin mixture known as lecithin; as this effect is indicated by part of the lecithin becoming less soluble, it is probable that this action of ether is due to the oxidising effect of some impurity in the ether on the kephalin part of the so-called lecithin. In one experiment bearing on this point, heart tissue was extracted with alcohol in the usual way, and the phosphatide taken up in ordinary good ether as supplied by the dealers. Another portion was treated in exactly the same way, ether from the same source being used, but only after careful purification. Using ordinary ether, a considerable amount of the phosphatide ultimately obtained was insoluble in alcohol, and part of the "lecithin" fraction became alcohol insoluble after a week. On the other hand, the specimen in which purified ether was used gave nearly all "lecithin" which when tested six weeks after preparation was found to be quite soluble in alcohol. From these anomalous results it is easy to explain the divergence of opinion with regard to the occurrence of kephalin in the tissues. Thus Erlandsen [1907] could find no trace of kephalin in heart muscle, while Koch and Woods [1905] found more kephalin than lecithin.

II.

Preparation of Kephalin [Parnas, 1909].

Parnas used petroleum ether for the preparation of kephalin on the ground that it extracts less of certain decomposition products of the tissue, and is obtained in a drier condition than ether. This investigator extracted his kephalin from brain according to the following plan:—

1. Fresh brain was rubbed up with acetone and made into a fine pulp. To the finely divided brain substance three times its weight of acetone was added and the whole left to stand for ten days; the hardened material was then separated from the acetone and spread out in a thin layer under a fan at 30°; this treatment dried it completely in less than one hour.

2. The dried mass was ground to a fine powder and extracted for a whole day with five times its weight of low boiling petroleum ether (b.p. 30 to 60°); the extraction was facilitated by the use of a shaking machine. After separation of the extract, fresh petroleum ether was added and the process repeated several times. The extracts were completely separated from residual material by the centrifuge. During the centrifuging a good deal of cooling naturally took place which caused the separation of large amounts of cerebrosides.

3. The combined extracts were concentrated at ordinary pressure as long as the temperature remained below 50° and then under diminished pressure. Some low boiling-point petroleum ether was then added; on strong suction being applied this easily evaporated, and as a result the mixture was cooled down to − 20° whereby large amounts of cerebrosides fell out.

4. The decanted liquid was further concentrated and precipitated with excess of alcohol. The precipitate was washed with alcohol, dissolved in petroleum ether and again treated with alcohol. The precipitate was then washed and dried *in vacuo*. This mass consisted of impure kephalin.

5. The kephalin was left to stand *in vacuo* for from ten to fourteen days; it was then powdered and shaken with five times its weight of cold ether. The mixture was centrifuged and the clear solution obtained left to stand in ice for twenty-four hours. After again centrifuging, the kephalin was precipitated by the addition of alcohol.

6. The kephalin was now purified by dissolving in ether and washing the solution with weak hydrochloric acid till calcium was no longer present in the acid. The ethereal solution was then washed several times with water, followed by weak soda and again by water. The purified kephalin was obtained from the ether solution by precipitation with alcohol.

III.

Preparation of Cuorin [Erlandsen, 1907].

Cuorin was prepared by Erlandsen from the ether extract of heart muscle. The heart was dried under a fan and extracted with ether in the usual way. The different steps were:—

1. The ether extract was freed from ether by evaporation at reduced pressure and the dried residue treated with a little fresh ether; the bulk of the extract passed into solution; a white insoluble residue was separated by filtration. To the concentrated clear ether solution acetone in excess was added, the precipitated phosphatide freed from acetone in the desiccator, again dissolved in ether and precipitated with acetone.

2. The dried material was dissolved in ether and excess of alcohol added; a precipitate immediately formed which was increased on allowing the mixture to stand in the cold (0° to 2°). The ether-alcohol mixture was separated, the precipitate washed with absolute alcohol, again dissolved in ether and precipitated with alcohol.

3. The alcohol-insoluble substance was treated with warm alcohol at 60° in an incubator whereby a good deal dissolved. The warm alcohol was decanted off and the residue dissolved in ether and precipitated with alcohol. The precipitate was once more treated with alcohol at 60°. On dissolving the residue in ether in an atmosphere of carbon dioxide at a low temperature a small amount of material separated leaving a clear solution. This solution was treated with acetone at 0°, the resulting precipitate separated by means of the centrifuge and dried.

4. The dried material was dissolved in a small amount of acetic ester which had been freshly distilled at 74° to 76°. On allowing to stand at a low temperature (about – 2°) a brown syrupy mass fell out. The treatment with acetic ester was repeated, the substance obtained freed from acetone and dried *in vacuo* over sulphuric acid. On analysis, this body was found to be cuorin.

Some Observations on the Occurrence of Kephalin and Cuorin.

As already mentioned there is some doubt as to the chemical identity of cuorin, particularly on account of its close resemblance to kephalin, both as regards physical properties and methods of preparation. There is no doubt whatever that a substance with a N : P ratio of about 1 : 2 can be obtained from several tissues and may be present in all tissues, but it remains to be proved that part of the phosphorus is not present as an impurity. At best, cuorin is present only in small amount, and when tissues are extracted under conditions where considerable oxidation can take place, large amounts of kephalin are obtained which would possibly obscure any cuorin present. No doubt some of this kephalin may be removed by treatment with hot alcohol, but it is equally certain that this does not happen under all circumstances. As previously pointed out, the greater care taken to use absolutely pure solvents and to avoid oxidation in the preparation of lecithin, the less kephalin is obtained. If the conditions are such as to yield much alcohol-insoluble substance it is not improbable that the so-called kephalin of all observers is really a mixture of kephalin with a small amount of cuorin. The predominance of the kephalin part might easily be of such an extent as not to affect materially the general analysis of kephalin, and it is noteworthy in this connexion that no kephalin yet obtained has on analysis furnished theoretical figures. From the very nature of the subject it is certain that cuorin can only be looked for under conditions much more rigid and expeditious than are at present generally applied by investigators.

IV.

Preparation of Sphingomyelin [Levene, 1914; 1916].

Sphingomyelin can be very easily obtained free from other phosphatides owing to its slight solubility in alcohol and ether, but its separation from cerebrosides which constantly accompany it in the tissue, is a matter of great difficulty. The isolation of sphingomyelin in a pure form was rendered much easier by the observation of Rosenheim and Tebb [1910, 1] that a mixture of sphingomyelin and cerebrosides could be separated by means of pyridine. This solvent is now invariably used in the preparation of sphingomyelin. A pure sample of this body should give no carbohydrate reaction on hydrolysis, and the absence of such a reaction is taken as an indication of its purity.

Levene [1914] gave explicit directions for the preparation of the pure substance, but showed later that the body obtained by this method was not quite pure. By a modification of his former process he now claims to be able to isolate pure sphingomyelin [Levene, 1916].

Since sphingomyelin contains no unsaturated fatty acids and does not appear to be influenced by exposure to air, it is not necessary to take such precautions in the drying of the tissue as is necessary in the case of the other phosphatides. Levene obtained pure sphingomyelin by the following steps :—

1. Desiccated brain tissue was exhaustively extracted with boiling alcohol, each extraction lasting about thirty minutes. On cooling this extract a precipitate formed.

2. The precipitate was thoroughly extracted with ether and acetone.

3. The residue was dissolved in hot technical pyridine and allowed to cool ; after standing for some time a precipitate formed.

4. The precipitate was dissolved in hot glacial acetic acid and allowed to cool when a deposit occurred. The mother liquor which contained sphingomyelin was concentrated under diminished pressure and transferred into acetone when crude sphingomyelin was precipitated.

5. The crude sphingomyelin was dissolved in a mixture of 5 parts ligroin and 1 part alcohol. Alcohol (98 per cent.) was added as

long as a precipitate formed. The filtrate was then allowed to stand overnight at 0° and again filtered. The final filtrate was concentrated under diminished pressure and poured into acetone.

The purity of the material obtained at this stage depended largely on the nature of the ligroin employed for solution, though the poorest specimens obtained were as pure as Thudichum's sphingomyelin.

6. The material obtained in this manner was further purified by recrystallisation from a solution consisting of equal parts of pyridine and chloroform. This was carried out in several stages. Five recrystallisations were made at room temperature; these were followed by recrystallisation at 30° and finally at 37°. The recrystallisations were continued until the substance gave a negative orcin test in the presence of a trace of copper acetate. The substance ultimately obtained was pure sphingomyelin.

CHAPTER IV.

THE CEREBROSIDES.

SYNONYMS: GALACTOSIDES (ROSENHEIM), CEREBRO-GALACTOSIDES (THUDICHUM), GALACTOLIPINES (LEATHES).

Nomenclature.

THE term cerebrosides was used by Thudichum [1874] to designate certain phosphorus-free lipin bodies of a glucoside nature which he had succeeded in isolating from the brain. On hydrolysis these bodies yielded amongst other products a reducing sugar, the nature of which Thudichum did not at first recognise. To this sugar he gave the name "cerebrose," and following the usual nomenclature of the glucosides suggested that these lipin compounds which furnished this sugar should be called cerebrosides. Later when it was recognised that "cerebrose" was really galactose, Thudichum suggested that the term cerebro-galactosides might be a more suitable name for this class of bodies. Recently Rosenheim [1913] proposed that the general term "galactosides" should be used to designate these substances. Since, however, the galactosides include a large number of bodies having no necessary connexion with lipins, it is of importance to have a somewhat more specific term to indicate these lipin products. Such a term is Thudichum's "cerebro-galactosides," but at best this is rather a clumsy combination and though useful is by no means an ideal designation. Similar objections apply to Leathes' "galacto-lipines". On the whole it appears best to retain the name cerebroside as originally used by Thudichum. Although these bodies are not restricted to the cerebral tissue, the fact that they are very abundant there and were originally obtained from brain, is sufficient reason for retaining their original name, at least for the present. Further, the fact that all the chief researchers in this field, with the exception of Rosenheim, use this term is another argument in its favour.

For our more recent knowledge of the cerebrosides we are chiefly indebted to the researches of Thierfelder [1890-1915] who was the first, after Thudichum, to undertake a systematic investigation of these substances. Valuable contributions have also been made by Levene and his collaborators [1912-1916] and by Rosenheim [1913-1916].

Historical.

The first phosphorus-free lipin extracted from the brain was isolated by Müller [1858] who called his product cerebrin. Previous to Müller's discovery the nearest approach to a substance resembling cerebrin was probably a body, containing 0·52 per cent. of phosphorus, obtained by von Bibra [1854]. Müller's cerebrin, which was entirely free from phosphorus, was isolated from brain which had been mixed with baryta water and boiled; the coagulum formed was extracted with hot alcohol, the alcoholic extract allowed to cool, and the resulting precipitate washed with ether to remove fat and cholesterol. The residue was again recrystallised from boiling alcohol when a light white powder without taste or smell, and having the empirical formula $C_{34}H_{33}NO_6$, was obtained.

Bourgoin [1874], by a process of fractional crystallisation at different temperatures, isolated a somewhat similar body from protagon; this product, however, contained less nitrogen than Müller's cerebrin. Köhler [1867] claimed to have obtained nitrogen-free cerebrins. Up to this time almost nothing was known as to the constitution or decomposition products of cerebrin, but in 1874 a remarkable paper by Thudichum appeared by which our knowledge of the phosphorus-free group of lipins, and incidentally of many other lipins, was materially advanced. This publication was so fundamental and far reaching that only in quite recent years has anything of moment been added to the observations which Thudichum then recorded. This work was probably the finest contribution ever made to lipin chemistry and yet for many years it was treated with cold neglect. Thus in a paper on cerebrin by Geoghegan [1879] five years later there is no reference to Thudichum's epoch-making results.

Thudichum recognised that the phosphorus-free bodies present in brain were analogous in their constitution to the vegetable glucosides, and distinguished two chief representatives of this class—phrenosin and kerasin.

Since phrenosin is relatively easy to prepare in large amount, Thudichum succeeded in elucidating its composition by a study of its hydrolytic cleavage products. Kerasin, though more difficult to isolate, was also very successfully investigated by him. It is important to note that recent research has established the fact that these two bodies are probably the only cerebrosides present in the tissues. Up to quite recent times, investigators paid too much attention to slight differences in the elementary composition or physical properties of cerebroside-like bodies obtained by them, with the result that the literature contains a copious list of supposedly new substances. These bodies which received such names as cerebrin [Müller, 1858; Parcus, 1881; Kossel and Freytag, 1893], homocerebrin [Parcus, 1881], entkephalin [Parcus, 1881], pseudo-cerebrin [Gamgee, 1880], cerebron [Thierfelder, 1904], *d*-cerebrine [Levene and Jacobs, 1912, 2] were nothing more than Thudichum's phrenosin or kerasin or mixtures of these two bodies. It must be admitted that some of these substances, such as Thierfelder's cerebron, were much purer products than Thudichum ever succeeded in isolating.

It is now customary, following the suggestion of Posner and Gies [1905] and of Rosenheim [1909], to designate the individual cerebrosides in terms of Thudichum's names—phrenosin and kerasin—rather than by any of the other terms mentioned.

GENERAL PROPERTIES OF THE CEREBROSIDES.

The cerebrosides are bodies of a glucoside nature which on hydrolysis furnish a reducing sugar (galactose), a base (sphingosine) and a fatty acid.

The fatty acid present in phrenosin differs from that in kerasin, a fact which helps to explain certain differences in the properties of these two closely related bodies [Thudichum, 1874; Rosenheim, 1916]. Their general similarity of composition is well seen by a comparison of their decomposition products.

Phrenosin
(1) Fatty acid = Phrenosinic acid ($C_{25}H_{50}O_3$).
(2) Carbohydrate = Galactose.
(3) Base = Sphingosine.

Kerasin
(1) Fatty acid = Lignoceric acid ($C_{24}H_{48}O_2$).
(2) Carbohydrate = Galactose.
(3) Base = Sphingosine.

These bodies possess many of the properties of sphingomyelin, particularly with regard to solubility in alcohol, a fact that renders the fractionation of mixtures of these products very difficult. Sphingomyelin is rather more soluble in cold absolute alcohol and less soluble in cold pyridine than the cerebrosides, a property which is taken advantage of in their separation. When dry, the cerebrosides are white and "more or less opaque, but are capable of becoming in part transparent like wax. They are deposited from alcoholic solutions in minute microscopic particles, which may be termed crystalline, but have no claim to be termed crystallised. These particles are arranged in various composite forms—balls or branched masses or rosettes" [Thudichum, 1884]. Under special conditions true crystals of *phrenosin* can be obtained [Wörner and Thierfelder, 1900; Rosenheim, 1914, 1], but so far no one has succeeded in obtaining true crystals of kerasin.

The cerebrosides dissolve easily in hot alcohol from which they are deposited on cooling. In cold absolute alcohol and in cold benzene they are practically insoluble; in hot benzene they dissolve easily, and are deposited on cooling as a gelatinous mass. The addition of cold alcohol to a hot benzene solution precipitates the cerebrosides in the form of white flakes. In ether, cold or hot, they are almost insoluble. They dissolve readily in pyridine at 30°, but are insoluble in cold acetone. From hot alcoholic or hot acetone solutions, the phrenosin constituent is first precipitated on cooling, and it is only at a temperature below that at which the most of the phrenosin separates that kerasin begins to fall out. The separation of the two cerebrosides from each other is based on this property.

The cerebrosides in common with sphingomyelin have the property of forming "liquid crystals" at certain temperatures, a phenomenon which accounts for many of the discrepant statements with regard to the melting-point of these substances recorded in the literature. [Rosenheim, 1914, 1.]

The cerebrosides are optically active bodies, phrenosin being dextrorotatory and kerasin laevo-rotatory.

On account of the presence of sphingosine and galactose in the molecule, the cerebrosides give certain colour reactions. Thus when rubbed up with concentrated sulphuric acid the particles gradually take on a deep purple-red colour, while in the presence of cane sugar and concentrated sulphuric acid the purple colour appears immediately.

The orcin reaction which is given by cerebrosides, as pointed out by Fränkel and Linnert [1910, 1], is dependent on galactose and not on the presence of pentose as these observers thought [Levene, 1907].

PHRENOSIN.

Thudichum obtained phrenosin by extracting brain with 85 per cent. alcohol at 45°. The "white matter" which deposited on cooling was treated with ether to remove lecithin and kephalin, and the residue recrystallised several times from alcohol. Further separation of such bodies as "myelin" and "cerebrinic acid" was carried out by treatment with lead acetate followed by ammonia and lead acetate until the hot alcoholic solution gave no precipitate on addition of these reagents. The product obtained at this stage was dissolved in hot absolute alcohol, and the processes of solution and recrystallisation repeated "a great number of times" until the mother liquor gave no further precipitate with cadmium chloride. The precipitate caused by cadmium chloride was due to sphingomyelin, which was to a large extent removed by this treatment. The residue now consisted largely of a mixture of phrenosin and kerasin which were partly separated by fractional recrystallisation. The mixture was dissolved in hot alcohol which on cooling began to deposit rosettes of phrenosin at from 50° to 40°. When the temperature reached 28° this precipitation ceased, and the supernatant liquor remained clear until the temperature fell to 26°. Below this temperature a gelatinous cloudy mass appeared which floated on the phrenosin and consisted mainly of kerasin. Isolation of phrenosin was therefore accomplished by allowing the hot alcoholic solution of mixed cerebrosides to cool to 28° when the precipitate which formed was separated. This product was mainly phrenosin, and on further recrystallisation in the same way gave a substance containing only 0·113 per cent. of phosphorus. Kerasin from the mother liquor had 0·198 per cent. of phosphorus. Both these substances also contained inorganic salts. Thudichum never succeeded in getting his products quite free from phosphorus despite the number of solutions and recrystallisations to which they were subjected. In the case of phrenosin, further treatment with cadmium chloride, ether and sulphuretted hydrogen decreased the phosphorus content to 0·048 per cent. Three analyses of this phrenosin gave the following results :—

No.	C.	H.	N.	O.
1	67·71	11·62	2·15	18·51
2	67·89	11·42	2·13	18·56
3	67·67	11·23	2·07	19·03
Average	67·75	11·42	2·11	18·70

Hydrolysis furnished as decomposition products a carbohydrate (cerebrose), a base (sphingosine) and a fatty acid (neurostearic acid).

Carbohydrate Radicle of Phrenosin.

Galactose = Cerebrose (Thudichum).

The "cerebrose" which Thudichum obtained from phrenosin by boiling with dilute sulphuric acid had the formula $C_6H_{12}O_6$ and separated in crystalline form. Previous to Thudichum's researches indications of the presence of a carbohydrate complex in certain brain lipins were obtained by Baeyer and Liebreich [1867] who showed that protagon yielded a sugar which had many of the properties of glucose. They considered that this sugar was present in glucoside formation.

Thudichum thought that cerebrose was a new hexose isomer, but it was shown by Thierfelder [1890] and immediately afterwards by Brown and Morris [1890] that cerebrose was identical with galactose.

Base of Phrenosin.

Sphingosine.

From phrenosin Thudichum [1881, 1] isolated a base, and identified it as a body of the composition $C_{17}H_{35}NO_2$; to this product he gave the name sphingosine (see p. 56). The correctness of Thudichum's observations was substantiated by Thierfelder [1904] who isolated sphingosine from cerebron; it was only after he had completely worked out the constitution of this base that Thierfelder became aware of Thudichum's observations. The results of these observers agreed very closely, and they both arrived at the same empirical formula. The presence of sphingosine in kerasin has been shown by several later observers.

Fatty Acid of Phrenosin.

Phrenosinic acid [Neuro-stearic acid (Thudichum), Cerebronic acid (Thierfelder)].

The fatty acid of phrenosin, like its other constituents, was first investigated by Thudichum [1881, 1] who isolated from the hydrolytic products of phrenosin a fatty acid resembling stearic acid. This product was separated in crystalline form and on analysis gave the following figures:—

C	75·88	per cent.
H	12·85	,, ,,
O	11·27	,, ,,

This percentage composition agreed very closely with that required for stearic acid ($C_{18}H_{36}O_2$), and its close relationship to stearic acid was further substantiated by an investigation of its corresponding ethyl ester which had the empirical formula $C_2H_5(C_{18}H_{35}O_2)$. So far the data obtained were the same as those furnished by stearic acid, but that the product was not stearic acid was recognised by Thudichum who showed that its melting-point was 84 to 85° as against 69·3° for stearic acid. This observer came to the conclusion that the acid of phrenosin was an isomer of ordinary stearic acid, and he designated it neuro-stearic acid.

Thudichum failed to recognise the real constitution of this acid, and to Thierfelder [1904] belongs the credit of having shown that Thudichum's neuro-stearic acid was really an hydroxy-acid of the formula $C_{25}H_{50}O_3$. This hydroxypentacosanic acid Thierfelder obtained from "cerebrone," and for this reason he called it "cerebronic" acid. Since "cerebrone" was simply a purer form of phrenosin than Thudichum had succeeded in isolating, there is no doubt that "cerebronic" acid and "neuro-stearic" acid were identical substances. Both products were prepared by similar methods, and Thierfelder's specimen yielded on analysis figures very close to those obtained by Thudichum:—

Cerebronic acid.
C = 75·33
H = 12·50
O = 12·17

In spite of this Thierfelder [1905, 1] declined to admit the identity of the two acids; for this view he had apparently good reasons, since cerebronic acid melted at 98 to 99° while neuro-stearic acid melted at 84 to 85°.

This anomaly was satisfactorily explained by Levene and Jacobs [1912, 1] who again obtained Thudichum's acid of low melting-point (82 to 85°). These observers showed that cerebronic acid exists in two optically isomeric states, of which the inactive *d-l* form melts at 82 to 85° and represents Thudichum's neuro-stearic acid, while the dextro-rotatory form melts at 106 to 108°. Thierfelder's cerebronic acid was therefore a mixture of the two forms in which the optically active compound predominated. The conditions under which these two isomers are formed have not yet been ascertained, and there does not appear to be any definite proof that the whole of the acid constituent of phrenosin is not originally present in the optically active form. In the isolation and hydrolysis of phrenosin, racemisation might possibly take place, so that in spite of certain results obtained by Levene and Jacobs [1912, 2], indicating that different isomers actually exist preformed in phrenosin, the matter is still undecided.

Rosenheim [1916] found that the melting-point of the dextrorotatory acid was lowered considerably by keeping it for some time in a toluene bath, and suggested that the change in melting-point might be due to the formation of an anhydride or lactone-like derivative. The presence of an hydroxyl group in the acid lends support to this view; this change, if it takes place, would explain the difficulties encountered by Levene and West [1913] in obtaining correct titration values for the inactive acid. Experiments to determine whether the decrease of the melting-point is accompanied by racemisation are being carried out by Rosenheim [1916].

The existence of these different isomers may explain the results of Grey [1913], who isolated from brain three hydroxy acids with different melting-points.

Thierfelder still refuses to admit the validity of Levene's observations, but maintains [Brigl, 1915] that the differences between cerebronic acid (m.p. *circa* 98 to 100°) isolated by him, and Levene's acid (m.p. *circa* 82 to 85°) are dependent not on stereo-isomerism but on *structural* isomerism. Thierfelder and Brigl adopt the view that phrenosin (cerebron) gives rise on hydrolysis to two structurally isomeric acids, one of which is cerebronic acid and the other an acid similar to the one obtained by Thudichum [1881, 1] and by Levene and Jacobs [1912, 1]. To this latter acid they gave the name neuric acid (neurosäure). According to this view phrenosin contains two optically active isomeric pentacosanic acids differing in structure.

This assumption is not in accord with the fact that the same sample of phrenosin yields a cerebronic acid of different melting-point and of different optical power according to the conditions of hydrolysis [Levene and West, 1916, 4; Rosenheim, 1916].

Further, Levene and West [1916, 4] showed that on oxidation with permanganate solution, both "cerebronic acid" (m.p. 99 to 100°, $[\alpha]_D^{20} = +2{\cdot}6$) and "neuric acid" (m.p. 86°, $[\alpha]_D^{20} = +1{\cdot}5$) yielded the same tetracosanic acid melting at 81°. If the original acids were different in structure, it is difficult to imagine that both would yield an identical derivative. For the present, therefore, it would seem that Levene and West's conception that "cerebronic" and "neuric" acids are structurally identical, and that the differences between them are dependent on optical isomerism, is the most probable explanation of the anomalies described.

Since the name phrenosin is now in general use it is best to call the acid derived from it *phrenosinic* acid [Posner and Gies, 1905; Rosenheim, 1914] instead of cerebronic or neuro-stearic acid.

Structure and Properties of Phrenosinic Acid.

Though Thierfelder showed that this acid had the empirical composition $C_{25}H_{50}O_3$, and that one hydroxyl group was present, he did not ascertain the position of this hydroxyl group, nor did he furnish any evidence as to the manner in which the carbon atoms were linked together. Indeed the percentage composition of carbon and hydrogen on which Thierfelder relied for his formula is of itself hardly sufficient, in the case of the higher fatty acids, to indicate the number of carbon atoms actually present in the molecule: the "acid value" of the substance furnishes a surer clue.

The position of the hydroxyl group was established by Levene and Jacobs [1912, 1] who showed that cerebronic acid when oxidised with alkaline permanganate solution [Edmed, 1898] gave rise to an acid of the constitution $C_{24}H_{48}O_2$. The hydroxyl group must therefore have been attached to the carbon atom next to the carboxyl group—the *a* carbon atom. That the carbon chain was an open or normal one was made probable by the transformation of phrenosinic acid into a hydrocarbon that melted from 54 to 57°.

According to Krafft [1882] the three hydrocarbons $C_{24}H_{50}$, $C_{25}H_{52}$, $C_{26}H_{54}$ have the melting-points 51°, 53·5° and 58° respectively. Since the melting-point of the hydrocarbon of phrenosinic acid was in the region of that obtained from the hydrocarbon $C_{25}H_{52}$, it seemed very probable that this substance also had the composition $C_{25}H_{52}$. This assumption was further substantiated by the fact that the acid value for pure phrenosinic acid corresponds to the molecular weight value of $C_{25}H_{50}O_3$ [Levene and Jacobs, 1912, 1].

In the light of this evidence, Levene and Jacobs at this time suggested that phrenosinic acid was the normal *a*-hydroxypentacosanic acid $CH_3 . (CH_2)_{22} . CH(OH) . COOH$, but later they modified this view. Thierfelder believes that cerebronic acid corresponds to the normal hydroxy acid, and in the hope of obtaining evidence in support of this contention Brigl [1915], working in Thierfelder's laboratory, prepared normal *a*-hydroxypentacosanic acid and compared its melting-point with that of cerebronic acid. The results were inconclusive, the figures obtained being as follows:—

	M.P.
(*a*) Cerebronic acid	= 100 to 101°
(*b*) *r*-Cerebronic acid	= 97 to 100°
(*c*) Synthetic n. α-Hydroxypentacosanic acid	= 102 to 104°
(*d*) Mixture of (*b*) and (*c*)	= 98 to 100°

Interesting evidence as to the nature of the carbon chain of phrenosinic acid was obtained by Levene [Levene and Jacobs, 1912, 1; Levene, 1913; Levene and West, 1913, 1913, 1], who showed that the acid $C_{24}H_{48}O_2$ obtained on oxidation of phrenosinic acid was really lignoceric acid, as it had the same melting-point and furnished the same derivatives [Hell and Hermanns, 1880; Kreiling, 1888] as this acid.

Since the melting-point of the hydrocarbon obtained on reduction of lignoceric acid was 51°, and agreed with that given in the literature for the normal tetracosane, Levene and West [1913, 1] concluded that lignoceric acid possessed a normal carbon chain.

About this time Meyer, Brod and Soyka [1913] showed that lignoceric acid was not identical, but isomeric with the normal tetracosanic acid, since a sample of the synthetical normal acid melted at 85·5 to 86° while lignoceric acid melted at 81°. This was corroborated by Levene, who prepared a similar normal product which melted at 87·5 to 88° [Levene and West, 1913, 1; Levene, West, Allen and Scheer, 1915].

Since Levene had based his view of the constitution of lignoceric acid on the similarity of the melting-point of the hydrocarbon derivative with that of the normal pentacosane (51°) [Marie, 1896] he set out to reinvestigate this point, and found that instead of melting at 51° the normal hydrocarbon melted at 55°. Levene therefore abandoned his original view of the structure of lignoceric acid and agreed that it was isomeric, but not identical with the normal tetracosanic acid.

The fact that lignoceric acid was easily derived from phrenosinic acid, indicated that phrenosinic acid also had a branched carbon chain [Levene and West, 1914, 1].

From Levene's observations on the relationship of lignoceric and phrenosinic acids it seems highly probable that phrenosinic acid ($C_{23}H_{47}.CH(OH).COOH$) is the *α*-hydroxy derivative of the next higher homologue of lignoceric acid. The point is not yet definitely settled, but Levene hopes to obtain conclusive evidence by converting lignoceric acid into an *α*-hydroxypentacosanic acid which on this view should be identical with phrenosinic acid, and finally to compare the pentacosanes obtained from phrenosinic acid and the synthetic pentacosanic acid [Levene and West, 1916, 4]. This, with some other suggestions indicated by Levene, should settle the matter, but it must be noted that up to the present Thierfelder's contentions as indicated by Brigl have by no means been absolutely disproved.

Phrenosinic acid is a snow-white easily powdered non-hygroscopic substance soluble in ether and in warm alcohol [Thierfelder, 1904]. It crystallises from alcohol or ether in characteristic "mamillary" or "cauliflower-like" masses [Thudichum, 1881, 1] which, under the polarising microscope, are seen to consist of irregular sphero-crystals. These crystals behave to the selenite plate test exactly as the mother substance phrenosin [Rosenheim, 1916].

As already indicated the acid exists in two conditions—the racemic and optically active forms.

Constitution of Phrenosin.

From the results of various observers it is now certain that phrenosin contains only the three components already mentioned, and possesses the empirical formula $C_{48}H_{93}O_9N$ [Thierfelder, 1905]. This contention is strongly supported by the figures obtained on elementary analysis. Three highly purified specimens of phrenosin examined by Rosenheim [1916] furnished the following data :—

No.	1.	2.	3.	Average.	Calculated for $C_{48}H_{93}O_9N$.
C	69·78	69·69	69·34	69·60	69·65
H	11·63	11·29	11·11	11·34	11·24
N	1·90	1·79	1·91	1·83	1·70

Though there are various ways in which these constituents of phrenosin might be linked together, the possible arrangements have been considerably narrowed down, with the result that a probable formula for this substance can now be given with a good deal of confidence.

The following observations relating to the individual components of phrenosin help to show the manner in which these different bodies must be linked together in the molecule.

1. The hydroxyl group of phrenosinic acid is free. This assertion is based on the fact that phrenosin forms a hexa-acetyl derivative, which contains one more acetyl group than the corresponding product

from kerasin. Since the only difference between phrenosin and kerasin is in the fatty acid, which in phrenosin has one hydroxyl group, it must be this hydroxyl group which gives the extra acetyl group. In order to do this the hydroxyl group must be free [Thierfelder, 1914, 1].

2. The NH_2 group of sphingosine $C_{12}H_{25} . CH : CH . CH (OH) . CH (OH) . CH_2 (NH_2)$ is not free, since when tested for free amino nitrogen in Van Slyke's apparatus no nitrogen could be obtained from cerebrosides [Levene and Jacobs, 1912, 2]. The fact that phrenosin has neither an acid nor an alkaline reaction is strong evidence that phrenosinic acid and sphingosine are united through the carboxyl and amino groups—$R . CO—NH . R'$—as in the case of the amino acids in protein [Thierfelder, 1914, 1].

From the fact that along with sphingosine some dimethylsphingosine is also formed during hydrolysis of phrenosin in methylic alcohol solution, it may be inferred that both the hydroxyl groups of sphingosine are at least partly combined with the other groups [Rosenheim, 1913, 1; 1916; Thierfelder, 1914, 1].

3. From our knowledge of the structure of the naturally occurring galactosides it may be assumed that, like these, phrenosin is a β-galactoside. Thus one of the hydroxyl groups of the sphingosine is combined through the aldehyde carbon of the galactose in the usual way [Rosenheim, 1913, 1]. That the aldehyde group of galactose is not present in the free form is evident, since phrenosin possesses no reducing properties [Thierfelder, 1914, 1].

According to Thierfelder, another of the hydroxyl groups of galactose is also in combination with sphingosine. This is unlikely, for if it were so we might expect to get some alkyl galactose on alcoholysis [Rosenheim, 1916].

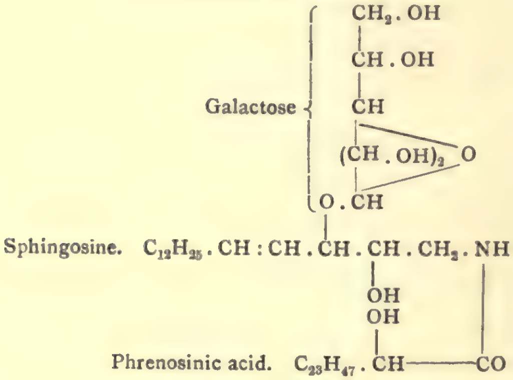
Rosenheim suggested that partial anhydride formation probably takes place between the sphingosine and phrenosinic acid hydroxyls, but that such linkage can only be partial is apparent from the fact already stated that the phrenosinic acid hydroxyl group is readily acetylated.

This assumption of anhydride formation furnishes a satisfactory explanation for the existence of the two modifications of phrenosin (p. 101). The ordinary "amorphous" phrenosin corresponds to the anhydride formula, while in the crystallised phrenosin the two hydroxyl groups are free. This view receives support from the observation that crystallised phrenosin contains 1 molecule of water more than the amorphous modification [Wörner and Thierfelder, 1900; Rosenheim, 1914, 1].

Rosenheim points out that if this view is correct, the completely crystallised phrenosin should give rise to a monoalkyl sphingosine, and suggests that a substance isolated by Thomas and Thierfelder [1912] from a sample of "cerebron" and which these observers first considered as a monomethyl-sphingosine did really contain monomethyl-sphingosine together with ordinary sphingosine.

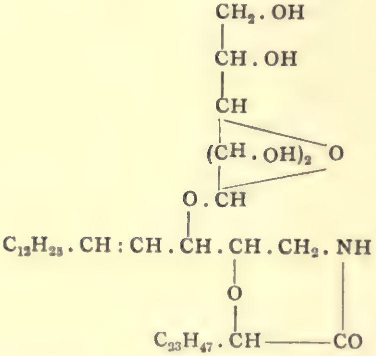
On the basis of these observations the following constitutional formula for phrenosin ($C_{48}H_{95}O_9N$) has been suggested [Rosenheim, 1916]:—

Crystallised Phrenosin.



$CH_2 . OH$
$CH . OH$
Galactose
CH
$(CH . OH)_2$ O
$O . CH$
Sphingosine. $C_{12}H_{25} . CH : CH . CH . CH . CH_2 . NH$
OH
OH
Phrenosinic acid. $C_{23}H_{47} . CH$ — CO

Amorphous Phrenosin.



Though these formulæ are in conformity with the results so far obtained in chemical investigations of phrenosin, they are by no means put forward as final. The results of partial hydrolysis of this compound, either by enzyme action or by other means, should furnish conclusive evidence as to the correctness of these formulæ; in this connexion it is interesting to observe that Thudichum long ago obtained evidence of the existence of hydrolytic intermediate compounds. One of these, which was galactose-free phrenosin, he called *æsthesin*, while to the other, which was phrenosin minus phrenosinic acid, he gave the name *psychosin*. Thierfelder [1914] also obtained galactose-free products from phrenosin. Phrenosin does not appear to be easily acted on by enzymes, for it resists the action both of pancreatic lipase and emulsin [Rosenheim, 1916].

Some Physical Properties of Phrenosin.

Liquid Crystals and True Crystals of Phrenosin.

In continuation of the work of Rosenheim and Tebb on protagon [1908] and of Lorrain Smith and Mair [1910, 1] on cerebrosides, Rosenheim [1914] showed that phrenosin can exist in the state of liquid crystals. Phrenosin, as usually obtained, consists of a white so-called crystalline powder, which does not possess any true crystalline form in the usual sense, but appears under the microscope as separate round, slightly anisotropic masses of more or less uniform size.

If a small quantity of this powder be heated on a slide on a Lehmann's polarisation microscope[1] until it is completely fused, it will be found to be invisible on examination in the dark field between crossed nicols. On allowing the slide to cool somewhat, the isotropic substance undergoes a change, and a shower of anisotropic needle-shaped crystals suddenly appears on the dark background. This appearance is caused by the formation of liquid crystals. On further heating, this liquid crystalline phase is transformed into the isotropic state. Phrenosin exists in this intermediate fluid crystalline state not only when the dry powder is heated, but also when it is allowed to separate from its hot solutions in various solvents.

Though phrenosin, as usually obtained, exists in the amorphous state, true crystals were obtained from cerebron [= phrenosin] by Wörner and Thierfelder [1900]. They found that when cerebron was kept at 50° suspended in 85 per cent. alcohol, or at 60° in methyl alcohol-chloroform [Loening and Thierfelder, 1910], the amorphous so-called "crystalline" product was gradually transformed into true crystals. Thierfelder did not realise the nature of this change, and assumed that it depended on intramolecular variations. This phenomenon, which Wörner and Thierfelder [1900] designated "umlagerung," was satisfactorily explained by Rosenheim [1914, 1], who showed that it consisted in the gradual change of the solidified liquid crystalline modification into the true crystalline condition at the temperature favourable for its formation.

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[1] A suitable electric hot stage has been described by Lorrain Smith and Mair [1910, 2].

Since the ordinary amorphous form of phrenosin consists of solidified liquid crystals, Rosenheim attempted to prevent the formation of this phase and so favour the deposition of true crystals. This can best be accomplished by allowing a hot 2 per cent. solution of phrenosin in 85 per cent. alcohol to cool very gradually in an unsilvered Dewar flask. When the flask is put into a water-bath at 75° and the temperature of the water-bath allowed to fall, the first crystals appear when the temperature reaches 65°, while at 61° the flask is filled with a mass of glittering crystals. Under the microscope these crystals appear as transparent well-defined plates resembling cholesterol. Crystallised phrenosin obtained from dilute alcohol contains 1 molecule of water of crystallisation which is not given up on drying *in vacuo* over concentrated sulphuric acid, but is removed on heating the substance at 105° [Rosenheim, 1914, 1].

Myelin Forms of Phrenosin.

In the presence of water phrenosin tends to give rise to myelin forms. The myelin forms of pure phrenosin were first observed in a preparation of Rosenheim's which was examined in Aschoff's laboratory [Kawamura, 1911]. Lorrain Smith and Mair [1910, 1] also observed that cerebrosides prepared by their method gave rise to similar formations. This phenomenon is intimately connected with the liquid crystalline condition, and is well seen when phrenosin is warmed with water on a slide under the microscope. Soon the small round masses swell up and are found to be covered with spicules which gradually develop into myelin forms. This takes place at a comparatively low temperature, and if the slide is allowed to cool it will be found that the phrenosin powder has taken up water and now appears as transparent globules. Under the polarising microscope the globules are strongly anisotropic and show the black cross of spherocrystals. With the selenite plate they behave exactly like the spherocrystals of phrenosin obtained from pyridine solution [Rosenheim, 1911, 1916].

Relation of above Phenomena to "Melting-Point" of Phrenosin.

The melting-points recorded in the literature for phrenosin show variations from about 170° [Parcus, 1881] to 212° [Wörner and Thierfelder, 1900]. Thierfelder's cerebron became moist at 130°, slightly yellowish at 200°, and fused at 209° on slow heating and at 212° on rapid heating. Similar variations were found by Rosenheim [1914, 1] who observes that it is possible by slight changes in the conditions to obtain what appears to be a melting-point at any temperature between 170° and 215°.

This behaviour is explained by the fact that phrenosin exists in the liquid crystalline condition between the temperatures of 95° and 215° approximately. The different statements as to the melting-point of phrenosin are apparently dependent on the fact that some observers considered the substance as fused during the liquid crystalline phase, while others took the higher temperature of the isotropic liquid phase as the melting-point.

There is really no true melting-point in the case of phrenosin, and the point at which it passes from the more viscid anisotropic liquid crystalline phase to the less viscid isotropic melt or liquid amorphous condition should not be termed its melting-point but its "clearing-" point [Lorrain Smith and Mair, 1910, 1; Rosenheim, 1914; Lapworth, 1913].

Optical Activity of Phrenosin.

Phrenosin is dextro-rotatory, the degree of its rotation being greatly influenced by the concentration, temperature and nature of the solvent. For investigations at ordinary temperatures pyridine is the most convenient solvent. "A 10 per cent. solution of phrenosin in pyridine, however, possesses at 20° only about half the optical activity of a 5 per cent. solution in chloroform-methyl alcohol at 40°, whilst a 10 per cent. solution in the latter solvent at 45° shows nearly three times the optical activity of a 10 per cent. pyridine solution at 20°" [Rosenheim, 1916]. It is therefore most important in recording observations on the optical activity of phrenosin that all the different factors should be stated.

As will be seen from the table, the results obtained by different observers agree fairly well with each other.

Observer.	Solvent.	Concentration. Per Cent.	Temperature.	Specific Rotation Obtained.
Kitagawa and Thierfelder [1906]	Chloroform methyl alcohol (3 : 1)	5	50°	$[\alpha]_D^{50^\circ} = + 6\cdot4^\circ - + 8\cdot4^\circ$
Thierfelder [1914, 2]	Chloroform methyl alcohol (3 : 1)	5	50°	$[\alpha]_D^{50^\circ} = + 7\cdot4^\circ$
Levene [1913, 1]	Chloroform methyl alcohol (3 : 1)	6	—	$\alpha(D) = + 9\cdot5^\circ - + 10\cdot7^\circ$
Rosenheim [1916]	Chloroform methyl alcohol (3 : 1)	10	45°	$[\alpha]_D^{45^\circ} = + 10\cdot4^\circ$
,, ,,	Chloroform methyl alcohol (3 : 1)	5	40°	$[\alpha]_D^{40^\circ} = + 7\cdot40^\circ$
Levene [1913, 1]	Pyridine	—	—	$[\alpha]_D = + 3\cdot05^\circ - + 4\cdot14^\circ$
Rosenheim [1916]	,,	10	20°	$[\alpha]_D^{20^\circ} = + 3\cdot78^\circ$
,, ,,	,,	10	20°	$[\alpha]_D^{20^\circ} = + 3\cdot70^\circ$
,, ,,	,,	10	18°	$[\alpha]_D^{18^\circ} = + 3\cdot70^\circ$
,, ,,	,,	10	30°	$[\alpha]_D^{30^\circ} = + 4\cdot30^\circ$

KERASIN.

Kerasin was the name applied by Thudichum [1874] to a second cerebroside which he succeeded in isolating from brain. A similar product was prepared by Parcus [1881] which he called *homocerebrin*. Following Thudichum, the term kerasin is now employed by common consent to indicate the second of the two cerebrosides present in brain. A substance of the nature of kerasin was investigated by Kossel and Freytag [1893].

Kerasin very closely resembles phrenosin in its composition and physical properties, and furnishes very similar figures on elementary analysis, so that much in the description of phrenosin applies also to kerasin. It differs from phrenosin in certain physical properties. Thus while phrenosin is deposited from hot solutions in alcohol and in other solvents in the form of rosettes, kerasin separates in a characteristic gelatinous form. On microscopic examination, this jelly-like mass is found to consist of balls composed of very fine long radiating needles. This gelatinous state is apparently due to the very long silky needles enclosing large amounts of solvent.

When kerasin is crystallised from absolute alcohol in a flask and allowed to dry slowly after the alcohol has been poured off, it becomes white and easily pulverised, so that it crumbles off the sides of the glass. When deposited from solvents containing water, it becomes, on drying, hard and waxy and difficult to powder.

Kerasin is only slowly deposited from its hot alcoholic solution, and if its amount does not exceed 1 part in 321 parts of alcohol, it is not deposited at all above 28° and below that temperature only very gradually [Thudichum, 1884]. It behaves somewhat similarly towards acetone and towards various other solvents and mixtures of solvents [Rosenheim, 1916]. The only successful method for its isolation in approximately pure form is based on this property.

Thudichum's Isolation of Kerasin.

The "white matter" obtained from brain in the manner already described (p. 92) was extracted with ether, dried, powdered and dissolved in hot absolute alcohol. An insoluble residue remained from which the hot alcohol was decanted. On standing, a deposit began to form which was filtered off after the first hour, while a further deposit was separated after the second hour. Next day the filtrate contained a gelatinous body which was separated. The solution was then allowed to stand for a few days in stoppered bottles, when a gelatinous membranous mass mainly consisting of kerasin was deposited. A further kerasin fraction was obtained from the filtrate by treatment with cadmium chloride which removed the sphingomyelin present. The kerasin was further purified by recrystallisation three times from boiling absolute alcohol; at this stage the alcoholic solution gave no precipitate with cadmium chloride or platinum chloride, so Thudichum considered that it was free from sphingomyelin; phrenosin, however, was still present.

The product was again recrystallised from pure absolute alcohol, and on examining the precipitate after three hours by the aid of the microscope it was found to consist of wavy crystallised masses of kerasin, while no rosettes of phrenosin were visible. At this stage the crystals were isolated, again recrystallised from alcohol and dried *in vacuo.*

The kerasin prepared in this way always contained small amounts of phosphorus (sphingomyelin?), the lowest phosphorus percentage found in a sample being 0·08 per cent. Very appreciable amounts of phrenosin were also present in all Thudichum's preparations. In spite of this, his investigations on kerasin were attended with a considerable amount of success.

The Composition of Kerasin.

On analysis kerasin prepared in the above manner furnished the following data from which Thudichum calculated the empirical formula of kerasin to be $C_{42}H_{85}NO_8$ or $C_{44}H_{89}NO_8$. The figures were obtained from different samples:—

No.	C.	H.	N.	O.
1	69·54	11·69	1·92	16·85
2	69·01	11·44	1·90	17·65
3	68·44	11·39	1·73	18·42
Average	68·99	11·50	1·85	17·64

It is interesting to note that these results obtained from a highly impure product agree very well with analyses of much purer specimens of kerasin, and do not differ materially from the theoretical numbers calculated for this product, namely, $C = 69·14$, $H = 11·49$, $N = 1·72$ [Rosenheim, 1916].

This observation furnishes a good instance of the unreliability of analytical results as an index of purity in the case of the cerebrosides. The difference in elementary composition between kerasin ($C_{47}H_{91}NO_8$) and phrenosin ($C_{48}H_{93}NO_9$) is so small that elementary analysis gives little or no indication of the presence of even a large amount of phrenosin in a kerasin fraction. Thus Thierfelder [1913] obtained a kerasin substance which gave almost theoretical analytical figures, and yet this kerasin contained such a large amount of phrenosin that on hydrolysis the fatty acid of phrenosin constituted 25 per cent. of the total fatty acids. So close is the resemblance between kerasin and phrenosin that Levene and Jacobs [1912, 2] originally considered them as optical isomers.

As the result of his experiments on kerasin, Thudichum concluded that kerasin, like phrenosin, was a *cerebroside*, which contained the sugar *cerebrose* combined with at least two other radicles. Of these, one was probably sphingosine, while the other was a fatty acid the nature and composition of which had not been perfectly ascertained. Later research has abundantly substantiated these statements of Thudichum, though it must be admitted that the experimental evidence on which they are based is much less complete than is generally found to be the case in the work of this investigator.

In extension of Thudichum's observations Thierfelder [1913] showed that kerasin consisted of 1 molecule of galactose,[1] 1 molecule of sphingosine and 1 molecule of an acid with the empirical formula $C_{24}H_{48}O_2$ which he called kerasinic acid. These results have been confirmed by Rosenheim [1916]. The only difference, therefore, between the constituents of kerasin and phrenosin lies in the fatty acid radicle.

Levene [1913] pointed out that Thierfelder's kerasinic acid was the same as lignoceric acid previously described by Hell and Hermanns [1880]. The nature of this acid has been already referred to and its relation to phrenosinic acid indicated: the fact that phrenosinic acid readily yields lignoceric acid on mild oxidation, throws an interesting light on the close connexion between the acids of the two cerebrosides. Of the structure of the carbon chain of lignoceric acid ($C_{23}H_{47}COOH$) little is known except that it is branched. On recrystallisation from a mixture of light petroleum and acetone, lignoceric acid is obtained in silky white crystals forming a loose powder which assumes a wax-like consistency when compressed in an agate mortar [Rosenheim, 1916]. Unlike phrenosinic acid this acid is optically inactive.

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[1] The galactose was not definitely identified until Rosenheim [1916] prepared the characteristic methylphenyl-hydrazone and showed that on oxidation mucic acid was produced.

Constitution of Kerasin.

The chief points on which the tentative constitution of kerasin is based are the same as those already discussed under phrenosin. It is assumed that kerasin is a glucoside [Rosenheim, 1913].

Kerasin when dried *in vacuo* at ordinary temperatures has the empirical formula $C_{47}H_{91}NO_8 + H_2O$ [Rosenheim, 1916]. On heating to 105° it gives up water. For samples of the purest kerasin hitherto isolated Rosenheim [1916] obtained the following figures :—

No.	1.	2.	3.	4.	5.	Average.	Calculated for $C_{47}H_{91}NO_8$ H_2O.
C	68·91	68·98	68·95	68·98	68·90	68·94	69·14
H	11·44	11·65	11·23	11·21	11·46	11·39	11·49
O	1·83	—	1·74	1·86	1·76	1·79	1·72

With the exception that kerasin forms a penta-acetyl derivative [Thierfelder, 1914] instead of the hexa-acetyl compound furnished by phrenosin, the chemical reactions of the two substances are very similar. Thus kerasin furnishes no nitrogen when treated with nitrous acid [Rosenheim, 1916 ; Levene and Jacobs, 1912, 2] and has neither an acid nor alkaline reaction [Thierfelder, 1914, 1]. On hydrolysis with mineral acids in aqueous solution, it furnishes galactose, lignoceric acid and a salt of sphingosine, while during methyl alcohol hydrolysis, monomethyl sphingosine and the methyl ester of lignoceric acid are formed [Rosenheim, 1916].

From these results it is obvious :—

1. That in kerasin one hydroxyl group of sphingosine is free.[1]
2. That kerasin contains five hydroxyl groups.
3. That the combination of sphingosine with fatty acid is through the amino and carboxyl groups as in phrenosin.

From consideration of these points Rosenheim [1916] proposed the following constitutional formula for kerasin :—

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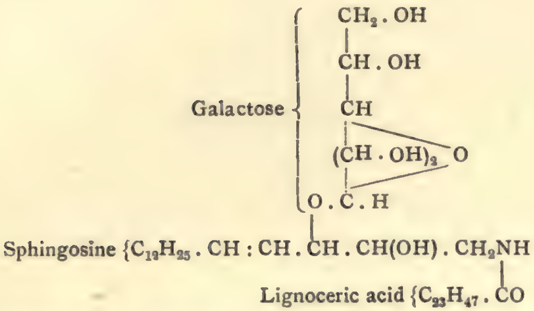
[1] The evidence as to the formation of monomethyl sphingosine during methyl alcohol hydrolysis is still inconclusive ; it is to be hoped that this will be definitely settled shortly, fo its bearing on the possible structure of phrenosin is obviously important.


Galactose
CH₂ . OH
CH . OH
CH
(CH . OH)₂
O
O . C . H
Sphingosine {C₁₃H₂₅ . CH : CH . CH . CH(OH) . CH₂NH
Lignoceric acid {C₂₃H₄₇ . CO

This formula is of course only put forward as a probable one, which may be either confirmed or shown to be untenable by future research. The possibility, in the case of both cerebrosides, of a linkage of sphingosine with galactose through the amino group must not be overlooked. It does not appear that the synthetic carbohydrate esters of the higher acids obtained by Bloor [1912, 1912, 1] have any bearing on the structure of cerebrosides.

Physical Properties of Kerasin.

In general physical properties, such as the formation of liquid crystals and myelin forms, kerasin is similar to phrenosin. Kerasin exists in the liquid crystalline state at temperatures from below 100° up to 180°, and like phrenosin it shows a "clearing"-point instead of a melting-point—i.e. a point at which the anisotropic liquid crystalline phase passes into the liquid amorphous melt. Though true crystals of phrenosin can be obtained, under certain conditions, as already described, true kerasin crystals have not yet been observed.

Optical Activity of Kerasin.

Thierfelder [1913] stated that a kerasin fraction obtained by him was inactive, and a similar observation was made by Levene and Jacobs [1912, 2]. These statements were due to the fact that the material used by the above investigators contained phrenosin. Many years ago Rosenheim and Tebb [1907] showed that kerasin was optically active and differed from phrenosin in that it was lævo-rotatory. Some of the latest figures obtained from the most highly purified specimens of kerasin hitherto examined are as follows [Rosenheim, 1916]. The observations were carried out in a tube 1 decimetre long. As in the case of phrenosin the degree of optical activity varies with the temperature, concentration and nature of the solvent.

No.	Solvent.	Concentration.	Temperature.	Specific Rotation Obtained.
1	Pyridine	Approx. 10 per cent.	20°	$[\alpha]_D^{20} = -2{\cdot}74$
2	,, ...	,, ,, ,,	18°	$[\alpha]_D^{18°} = -2{\cdot}78$
3	,, ...	10 per cent.	18°	$[\alpha]_D^{18°} = -2{\cdot}50$
4	,, ...	Approx. 10 per cent.	25°	$[\alpha]_D^{25°} = -3{\cdot}71$
5	Chloroform and 10 per cent. pyridine	,, ,, ,,	50°	$[\alpha]_D^{50°} = -5{\cdot}08$
6	Pyridine	,, ,, ,,	25°	$[\alpha]_D^{25°} = -3{\cdot}78$
7	Pyridine and acetone (1 : 1) .	,, 5 ,,	50°	$[\alpha]_D^{50°} = -4{\cdot}58$

Preparation of the Cerebrosides.

In the preparation of the cerebrosides the two main points to be considered are:—

1. The separation of the cerebrosides from accompanying phosphatides and cholesterol.

2. The separation of the purified cerebroside mixture into its components—phrenosin and kerasin.

For the preparation of cerebrosides the best material is brain. The means adopted for the separation of the cerebrosides from the other brain lipins may be divided into two groups. In the older methods generally, phosphatides were separated from cerebrosides by purely physical means, chiefly by the use of alcohol [Thudichum, 1884], glacial acetic acid [Koch, 1904] and other solvents or mixtures of solvents. Since the removal of certain phosphatides was accomplished by this method only with the greatest difficulty and sometimes very imperfectly, certain observers took advantage of the relative resistance of cerebrosides to saponification. They boiled the mixture with baryta for a short time, and thus destroyed the phosphatides while the cerebrosides were left intact. The resulting hydrolytic mixture contained the degradation products of the phosphatides together with the cerebrosides which were now more easily separated in the pure state.

In the baryta method the brain itself was sometimes boiled with the baryta [Müller, 1858; Parcus, 1881], and the cerebrosides subsequently extracted, while in other cases the residue obtained on cooling an alcoholic extract of brain was employed. This method which was originally introduced by Müller [1858] has been used, with certain slight modifications, by many recent observers: Kossel and Freytag [1893]; Loening and Thierfelder [1911]; Smith and Mair [1910, 1911, 1913, 1], Fränkel [1911], Lapworth [1913].

There can be no question that preliminary treatment with baryta materially facilitates the isolation of the cerebroside mixture, and the only possible argument against its use is the fact that cerebrosides themselves are not entirely resistant to the prolonged action of aqueous or alcoholic baryta solutions. This does not appear to be of much importance from the practical standpoint for prolonged boiling is not required, and Loening and Thierfelder [1911, 1912] showed that cerebrosides were apparently not injured by boiling for short periods with baryta, or even with methyl alcohol solutions of potassium hydroxide. On the other hand, too prolonged boiling might possibly give rise to difficulties, and in this connexion it is perhaps significant that only those observers who used the baryta method found a high nitrogen percentage in their products. This fact suggests that their preparations contained certain amounts of the nitrogenous base sphingosine derived from sphingomyelin, or perhaps from the cerebrosides themselves [Rosenheim, 1914].

Even with the help of baryta, great difficulties are experienced in the isolation of phosphorus-free cerebrosides, chiefly due to the fact that alcohol, which was universally used for recrystallisation, is unsuitable. This difficulty was to a great extent overcome by Lorrain Smith and Mair [1910] and by Loening and Thierfelder [1911] who, working independently, replaced alcohol by acetone. By this modification it is easy to obtain pure cerebrosides from brain, but in all cases the product obtained is a mixture of the two cerebrosides—phrenosin and kerasin.

Recently what appears to be an improvement in the methods of isolating cerebrosides has been introduced by Rosenheim [1914], who extracts these substances directly from the brain by means of pyridine. The mixture so obtained is separated into its constituents by temperature fractionation from pure acetone.

Observations on the Separation of Phrenosin and Kerasin.

The separation of these two bodies from each other is a troublesome matter, the chief causes of the difficulty being dependent on the following points [Rosenheim, 1914]:—

1. The close resemblance in their chemical constitution.
2. The close resemblance of their physical properties—solubility, etc,

3. Their chemical inertness, which prevents the formation of derivatives suitable for their purification.
4. The fact that they form liquid crystals, which according to O. Lehmann are easily and readily miscible.
5. The absence of any criterion of purity by which the completeness of the separation can be judged. Elementary analysis and melting-point determinations are of no value in this respect and indeed may even prove misleading.

The actual isolation of phrenosin and kerasin from a mixture of these cerebrosides can be accomplished by the method of temperature fractionation from alcohol introduced by Thudichum [1884] or by a modification of this method in which acetone is used [Rosenheim, 1914]. No method except that of fractionation, as suggested by Thudichum, has proved of any value in the separation of these products.

For indicating the progress of the separation of the cerebroside mixture two observations made by Rosenheim and Tebb [1908] are of considerable value :—

1. Phrenosin in pyridine solution is dextro-rotatory, while kerasin is lævo-rotatory in the same solvent.

2. The behaviour of the two substances under the polarising microscope is characteristically different. This fact permits of the detection of phrenosin in presence of kerasin, and *vice versa*, by a simple test known as the selenite-plate test.

Selenite Plate Test [Rosenheim, 1914].

On gradually cooling a warm 10 per cent. solution of phrenosin and kerasin, both these products separate in the form of sphero-crystals. If these crystals are now examined by means of a polarisation microscope with crossed nicols, and if a selenite plate is placed below the stage and immediately above the polariser in such a way that its axis lies diagonally to the plane of polarisation of the crossed nicols, a characteristic difference between phrenosin and kerasin is at once seen. The sphero-crystals now appear to be divided into quadrants of which the opposite ones show the addition colour blue, whilst the two others show the subtraction colour yellow. The important point is that while the sphero-crystals of phrenosin show the blue colour in the upper right and left lower quadrants, the sphero-crystals of kerasin show the blue colour in the reverse positions—in the left upper and right lower quadrants. By means of this simple physical test, it is possible to indicate in a very small amount of material when a pure sample of phrenosin or kerasin has been obtained. That the test is sufficiently sensitive for practical purposes follows from the fact that a kerasin fraction which was found to be free from phrenosin by this test gave on hydrolysis, no trace of phrenosinic acid, the typical fatty acid of phrenosin.

The general principles involved in the isolation of cerebrosides will be seen from the description given below of the two chief methods now in use—(1) The baryta method in combination with acetone as used by Thierfelder, and (2) Rosenheim's pyridine method. The direct extraction and isolation by means of alcohol as employed by Thudichum has been already described.

I. Isolation of Cerebrosides by Baryta and Acetone Method.

Loening and Thierfelder [1911] obtained cerebrosides from brain in the following manner. The brain was dried and treated with ether. The residue was then extracted with alcohol at 45°, and the precipitate ("protagon") obtained on cooling used for the preparation of cerebrosides. A known weight of this protagon was rubbed up with saturated baryta water, the mixture placed in a flask, mixed with six times its volume of this fluid and heated on a boiling water-bath for one to two hours. The flask now contained a precipitate and a clear yellowish supernatant fluid. The precipitate was separated by means of the suction pump, washed with water to remove traces of barium and finally with cold acetone to remove water. It was then extracted many times with boiling acetone; in the first extraction, boiling was continued for a few minutes only but in the later ones for several hours. From the first acetone filtrate a well-marked precipitate deposited on cooling: the amount of precipitate from subsequent extracts gradually became less and less, until in the case of the final extracts nothing separated unless the mixture was cooled in ice-water and, even then, only traces were obtained. The whole of the cerebrosides, however, could not be extracted by this method even after boiling with acetone for from eight to twelve hours, but the final extract contained so little, that it hardly influenced the yield when the method was used quantitatively. The precipitates, with the exception of the first which was slightly coloured, were pure white. All the different precipitates were mixed, dried and weighed. The mixture was found to be entirely free from phosphorus and barium. Separation of the two components of this cerebroside mixture was brought about by the employment of Thudichum's temperature fractionation method.

An excellent description of a similar method for isolating cerebrosides is given by Lorrain Smith and Mair [1910, 1913, 1] who worked out and used this method before Thierfelder's publication appeared.

II. Isolation of Cerebrosides by Rosenheim's Pyridine Method.

The method consists in the employment of the various steps indicated below. The crude cerebrosides are first separated, and the cerebroside mixture afterwards divided into its constituents.

A.

Preparation of Crude Cerebroside Mixture [Rosenheim, 1913].

1. Treatment of Brain with Acetone.

The finely minced ox brain was suspended in acetone and allowed to stand with frequent stirrings for twenty-four hours at room temperature. The watery acetone extract was decanted, and the brain pulp strained through several layers of fine muslin. At least six subsequent extractions with acetone were made, until the last extract yielded on evaporation only an inappreciable amount of cholesterol.

The tissue was next spread in a thin layer on a large glass plate, gently warmed from below, and freed from acetone by means of an air current generated by an electric fan. The brain tissue was now practically free from water and cholesterol, and much fat and fatty acids were removed.

2. Cold Petroleum Ether Treatment.

The somewhat waxy powder was extracted with cold petroleum ether to remove the unsaturated phosphatides (lecithin, kephalin and cuorin). The residue, freed from petroleum ether in the manner described above, was passed through an Excelsior mill whereby a fine cream-coloured powder was obtained.

3. Isolation of Crude Cerebrosides by Pyridine.

This powder was treated with pyridine and the mixture warmed to 45° to allow the solvent to penetrate the tissues; it was then rapidly cooled to room temperature and filtered by means of a Buchner funnel. From the pyridine filtrate, the cerebrosides were obtained by pouring it into 3 to 4 volumes of acetone, when a bulky precipitate at once formed. In order to increase the yield the pyridine-acetone mixture was cooled to 0°, and the precipitate separated by filtration through a plain filter paper. It was then thoroughly washed with acetone, removed from the filter paper and suspended in excess of acetone. Finally the product was extracted with ether in a Soxhlet apparatus to remove the last traces of ether-soluble phosphatides.

The crude cerebrosides thus obtained presented a slightly yellowish appearance, but after being twice recrystallised from 15 volumes of an alcohol-chloroform mixture (1 : 2) were pure white. The substance contained 1·68 per cent. of nitrogen and was almost phosphorus-free containing only ·08 per cent.

B.

Separation of Phrenosin and Kerasin from Crude Cerebroside Mixture [Rosenheim, 1914].

Rough Separation by Acetone into two Fractions.

The finely powdered cerebroside mixture was treated with excess of acetone containing 10 per cent. of water, on a water-bath kept at 56°. About 85 per cent. of the product went into solution. The clear acetone solution was allowed to cool in an incubator at 37°. After sixteen to twenty hours a crystalline deposit had formed from which the supernatant fluid was decanted through a filter warmed to 37°. This crystalline deposit consisted chiefly of phrenosin, and is referred to later as the *phrenosin fraction.*

On allowing the filtrate to stand in an ice chest for twenty-four hours or longer, a milky gelatinous precipitate was obtained which was chiefly kerasin and is considered as the *kerasin fraction.*

The precipitates were filtered under pressure, washed with acetone and dried *in vacuo.*

Phrenosin.

1. *Isolation of phrenosin from phrenosin fraction by glacial acetic acid-chloroform mixture* (3 : 2).

The phrenosin fraction was dissolved in 4 volumes of chloroform at about 60°, and to the solution were added 6 volumes of glacial acetic acid previously warmed to the same temperature The clear solution was kept in an incubator at 37° over night, the deposit filtered and washed at 37° with acetic acid-chloroform mixture.

The moist precipitate was again dissolved in the mixture and the solution heated as before. The mother liquors, from these two recrystallisations, deposited at room temperature a considerable amount of gelatinous precipitate which was worked up with the kerasin fraction.

The product obtained at 37° was granular and easy to filter. When recrystallised a third time in this way, the mother liquor no longer gave a deposit on cooling to room temperature.

On examination by the selenite plate only a small quantity of kerasin was found to be present at this stage. The product was entirely free from phosphorus.

Since no further separation of kerasin could be accomplished by means of the acetic acid-chloroform mixture, another solvent, acetone-chloroform, was used.

2. *Further purification of nearly pure phrenosin with acetone-chloroform mixture* (3 : 2).

The product was dissolved in 4 volumes of chloroform and 6 volumes of warm acetone added. The granular deposit which formed was filtered at 37° and twice more recrystallised in the same way. The substance now obtained appeared to be perfectly uniform, since no indication of the presence of kerasin was obtained on examination by the selenite plate. Finally, the phrenosin was recrystallised from a large volume of acetone containing 10 per cent. of water. The product obtained in this way appeared to consist of pure phrenosin.

Kerasin.

1. *Isolation of kerasin from kerasin fraction of glacial acetic acid-chloroform mixture* (3 : 2).

The kerasin fraction described above was dissolved in 4 volumes of chloroform at 50°, and to the solution were added 6 volumes of glacial acetic acid previously warmed to about 60°. When kept at 37° a granular white layer mainly consisting of phrenosin collected on the surface and was filtered off. The filtrate began to deposit on cooling to 26° and solidified finally to a gelatinous mass. After filtering and washing with the acetic acid-chloroform mixture the product was suspended in acetone. On filtration it was again recrystallised from acetic acid-chloroform mixture as before. A small amount separated at 37°, but the main quantity deposited at room temperature. The process was repeated twice more, when it was found that the solution no longer furnished a deposit at 37° even when kept at that temperature for many hours. At this stage the selenite plate showed that the substance consisted almost entirely of kerasin.

2. *Further purification of nearly pure kerasin by acetone-pyridine mixture* (1 : 1).

The product was dissolved in 10 volumes of pyridine to which 10 volumes of acetone warmed to 45° were added. On cooling to 37° in the incubator only a faint cloud of phrenosin appeared. The filtrate began to deposit at 28°, and was allowed to cool to room temperature before filtration. This process was repeated, and the product finally recrystallised from a large volume of 90 per cent. acetone containing 2 per cent. pyridine. The kerasin so obtained appeared to be pure.

Occurrence of Cerebrosides in the Different Organs and Tissues.

Though brain and also nerve material [Chevalier, 1886] constitute the chief sources of the cerebrosides, products identical with, or similar to, the cerebrosides have been isolated from many other organs and tissues. It is not improbable that all adult animal organs contain at least traces of these substances, and their presence must be assumed in all organs yielding so-called protagon. The presence of cerebrosides has been established with more or less certainty in kidneys [Dunham, 1905; Rosenheim and MacLean, 1915]; heart [MacLean, 1913]; retina [Cahn, 1881]; thymus [Lilienfeld, 1893]; adrenals [Rosenheim and Tebb, 1909, 2]; liver [Waldvogel and Tintemann, 1904]; egg yolk [Stern and Thierfelder, 1907]; red blood corpuscles [Bang and Forsmann, 1906]; pus [Hoppe Seyler, 1871; Kossel and Freytag, 1893]; adipocere and spermatozoa [Kossel and Freytag, 1893]; sputum [Schmidt and Müller, 1898]; mushrooms [Bamberger and Landsiedl, 1905; Zellner, 1911] and other tissues.

It is interesting to note that cerebrosides appear to be absent in the brain of the fish [Argiris, 1908]. The distribution of cerebrosides in the developing brain is described by Lorrain Smith and Mair [1912, 1913].

On Various Names which have been Used to Designate the Cerebrosides.

Though various names have been employed by different observers to designate cerebroside bodies, it is now generally accepted that all these substances correspond to more or less pure phrenosin or kerasin.

The relationship of these differently named substances to each other has been the subject of discussion by such observers as Thierfelder [1904], Posner and Gies [1905], Fränkel [1909], Leathes [1910], Bang [1911], Cramer [1911], Levene and Jacobs [1912, 2], Rosenheim [1916] and others.

From a general survey of the whole question, especially in the light of more recent work, there seems little room for doubt that these bodies are all identical with Thudichum's phrenosin or kerasin as shown in the following list. Some of them were obtained in a purer state, others in a less pure state, than Thudichum's substances.

PHRENOSIN	Cerebrin [Müller, 1858 ; Koch, 1902 and others].
	d-Cerebrin [Levene and Jacobs, 1912, 2].
	Pseudocerebrin [Gamgee, 1880].
	Cerebrone [Wörner and Thierfelder, 1900].
	Pyosin [Kossel and Freytag, 1893].
	Amino-cerebrinic acid glucoside [Bethe, 1902].
KERASIN	Homocerebrin [Parcus, 1881].
	Pyogenin [Kossel and Freytag, 1893].

Of the various substances isolated by Thudichum to which he gave the names aesthesin, psychosin, krinosin, breginin, cerebrinic acid and sphaerocerebrin, some, such as aesthesin and psychosin were undoubtedly decomposition products, and it is probable that the majority of these bodies come under this category.

As regards krinosin and breginin it is impossible to make any definite statement, and their existence as preformed chemical units in the brain, though unlikely, is by no means excluded.

Quantitative Estimation of Cerebrosides.

The earlier attempts at the quantitative estimation of cerebrosides in brain were generally carried out by the method introduced by Noll [1899] in which the "protagon" from brain was hydrolysed, and the amount of reducing sugar so obtained estimated by Fehling's solution [Koch, 1904]. The yield of sugar afforded some clue to the amount of cerebrosides present, but the method did not give very satisfactory results. Falk [1908] attempted to separate the cerebrosides and weigh them as such, but without much success. In more recent times Lorrain Smith and Mair [1911; 1913, 1] have carried out some careful quantitative estimations, which no doubt yielded the first reliable results we possess. These observers separated the cerebrosides from other lipins with baryta and weighed them directly. From their figures it appears that the percentage of cerebrosides in normal human brain is about 1·6 per cent. in wet and 7·3 per cent. in dried brain.

Rosenheim [1916] isolated cerebrosides by the method described above and obtained 2 per cent. for wet brain and 9 per cent. for dry brain. These figures, which were obtained by weighing a crude product, agree fairly well with those of Lorrain Smith and Mair.

CHAPTER V.

PROTAGON.

In describing sphingomyelin it was stated that this phosphatide is obtained in the crude state when brain or other animal tissue is extracted with hot alcohol. On cooling the alcoholic extract, a whitish precipitate forms, which contains sphingomyelin and cerebrosides along with small amounts of other substances. To the part of this crude precipitate which remained after extraction with ether Liebreich [1865] gave the name protagon. Protagon "is nothing more than the solid part of an alcoholic extract of the brain, the more soluble portions of which solid part have been washed away with ether" [Thudichum, 1896]. For the last fifty years the name "protagon" has from time to time appeared, particularly in connexion with investigations on brain chemistry, but from the nature of the subject only a comparatively small number of chemists have taken any interest in it, and to many the name may have appeared unintelligible. To some, according to Thudichum, "it was so much so that they believed it to be that of an animal". This substance, however, has given rise to more bitter and acrimonious discussion among certain investigators than any other of the lipin bodies, and much energy has been expended by different observers in endeavours to ascertain whether protagon is a chemical unit or a mixture.

The question after all does not appear to be of supreme importance, but to the various "Protagonists," who have from time to time come forth in support of their contentions, it seemed to be a point of material moment, and so zealously did the different schools affirm their opposing views that on more than one occasion the discussion degenerated into something rather more personal than scientific.[1]

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[1] See for instance Thudichum [1881] in "Annals of Chemical Medicine," **2**, p. 183, where he makes a fierce attack on Gamgee. With regard to Gamgee's action Thudichum says: "Dr. Gamgee has published the most violent of these attacks without his name being attached thereto. However, in one of the German versions of his and Blankenhorn's paper on 'protagon,' he applies to Thudichum's researches epithets of which the English equivalent is 'cooked'."

Nature of Protagon.

Protagon is a white non-hygroscopic product which separates from solvents in the form of more or less well-defined crystals; when a warm alcoholic solution of protagon is quickly cooled, beautiful rosettes and needles are obtained. After drying *in vacuo* it forms a light white powder. Protagon is practically insoluble at room temperature in the majority of the ordinary organic solvents, but dissolves in warm pyridine, methyl alcohol, ethyl alcohol, glacial acetic acid, acetone and in various mixtures of solvents. On cooling these solutions precipitation takes place: protagon is practically insoluble in cold or boiling ether. The principal solvent used in investigations on protagon is alcohol: from hot or boiling alcohol in which the substance is easily soluble, deposition readily takes place on cooling, and this property is made use of extensively in the isolation of protagon.

Protagon which has been dried *in vacuo* is much less soluble in hot alcohol than moist (hydrated?) protagon [Posner and Gies, 1905, 1]. A somewhat similar behaviour is exhibited towards other solvents such as hot acetone, in which protagon after drying becomes much less soluble than it was previously [Rosenheim and Tebb, 1909, 1]. This curious behaviour appears to be characteristic of certain lipin substances as pointed out by Bang and Forsmann [1906].

All investigators are now agreed that the chief constituents of protagon are:—

1. Cerebrosides (phrenosin and kerasin),
2. Sphingomyelin,

while small amounts of other substances are also present. The crystalline appearance of protagon, together with the fact that under given conditions the substance can be recrystallised from alcohol without any appreciable change in its composition, strongly suggests at first sight, that the body is a definite chemical compound. This question is fully discussed below, and evidence advanced which must be accepted as proving definitely that protagon, despite the superficial characteristics mentioned, must be regarded as a mechanical mixture. This mixture is composed of the difficultly soluble cerebrosides and sphingomyelin in such proportions that the product usually contains about 1 per cent. of phosphorus. Although the brain yields large amounts of protagon it may be obtained from other organs. According to Thudichum [1879, 1880, 1880, 1], Rosenheim and Tebb [1909] and others, protagon always contains potassium: this, however, is denied by Gamgee [1880, 1], Roscoe [1880, 1880, 1][1] and Cramer [1911]. Sulphur is generally present, but it is not known in what form it exists: iron has been found by some observers [Rosenheim and Tebb, 1909]. No purpose would be served in enumerating certain alleged physical constants of this mixture such as its melting-point. Some important optical phenomena exhibited by protagon and dependent on its sphingomyelin constituent will, however, be considered here.

The name protagon has entirely lost its original significance, but is useful in designating certain mixtures of cerebrosides and sphingomyelin obtained from organs by extraction with hot alcohol and other solvents. For this purpose it is perhaps worth while retaining a name which has ceased to have any meaning in the chemical sense, but is of some importance from the practical point of view. The tremendous amount of literature which has sprung up in connexion with this substance also renders it advisable to retain the name protagon at least for the present, otherwise confusion might ensue.

Some Optical Phenomena Associated with Protagon.

Rosenheim and Tebb [1907] showed that protagon was optically active, an observation which was confirmed by Wilson and Cramer [1908]. When dissolved in pyridine in 3 per cent. solution and examined at 30° protagon possesses a slight dextro-rotation, which when expressed in terms of specific rotation amounts to about $[\alpha]_D^{30} = + 6{\cdot}8$. On raising the temperature this dextro-rotation decreases till at 50° optical inactivity results. The same phenomenon is observed when the temperature is allowed to fall below 30°. During cooling a slight haze is observed, due to partial deposition of the sphingomyelin of the dissolved product, and simultaneously with the formation of this deposit the fluid becomes strongly lævo-rotatory. This lævo-rotation finally reaches a maximum value which when expressed as $[\alpha]_D$ is equal to $- 100$ to $- 116°$: the temperature at which this maximum result is observed is about 15 to 20°. At this stage observation is rendered difficult by the opacity of the fluid. As the precipitate settles the lævo-rotation gradually decreases, until after a period varying from a half hour to three hours the clear supernatant fluid shows a constant rotation of $[\alpha]_D = - 13{\cdot}3°$. On shaking the contents of the tube and thereby distributing the precipitate in the fluid, the remarkable fact is observed that the lævo-rotation reaches the high value of about – 232°. On again heating the tube to 30° the original dextro-rotation of 6·8° is obtained[1] [Rosenheim and Tebb, 1908, 1].

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[1] In his second communication, Roscoe states that he found 0·0236 per cent. of potassium in a sample of Gamgee's protagon.

In the course of an investigation into the cause of these curious changes Rosenheim and Tebb [1908, 3] succeeded in elucidating several important points bearing on the alleged unit nature of protagon, while at the same time a new physical phenomenon was discovered. These observers examined the precipitate formed on cooling a protagon solution, as well as the supernatant fluid, and found that the precipitate consisted largely of impure sphingomyelin. Investigation of this phosphorus-rich deposit in the polarimeter proved that the peculiar behaviour of protagon in polarised light was mainly due to this constituent of protagon. When this sphingomyelin deposit was dissolved in pyridine and its optical activity investigated it was found to exhibit the same phenomena as protagon. The supernatant fluid, as already stated, showed in most cases an optical activity of $[a]_D = -13·3°$. After filtration the clear pyridine solution obtained was found to be inactive. When carefully pipetted off so that it appeared perfectly clear its optical activity was as before $[a]_D = -13·3°$. By filtration through paper it had lost its optical activity. From this it appeared that the lævo-rotation observed must have been due to a substance in suspension, an assumption which was proved to be correct by the observation that the lævo-rotatory clear fluid became inactive after centrifugalising.

We have here the first case described in the literature in which an optically active "solution" becomes inactive on centrifugalising owing to the removal of suspended particles. The explanation of this phenomenon was found in the peculiar physical structure of these particles, which Rosenheim and Tebb succeeded in demonstrating by means of the polarising microscope.

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[1] Rosenheim and Tebb have pointed out that the calculation of the observed rotation as $[a]_D$ is not justified in the case of protagon because it is not a definite chemical compound, and also because the constituent to which its optical activity is principally due does not follow the laws of optical activity as expressed by Biot. A calculation of the results as $[a]_D$ is, however, of value for comparative purposes. The exact figures obtained with different protagons vary somewhat and depend on the phosphorus percentages of the products. The results quoted here were obtained with a protagon containing about 1 per cent. of phosphorus.

This Phenomenon is produced by "Fluid Sphero-crystals" of Sphingomyelin.

When the sphingomyelin precipitate, obtained by cooling a pyridine solution of protagon, is examined under the microscope in ordinary light it is seen to consist of small globules resembling fat globules, but owing to their transparency they are difficult to distinguish. Their spherical form is, however, well seen in dark illumination. Further examination of these globules between the crossed prisms of a polarising microscope shows them to be strongly anisotropic, standing out brightly on a black background and being divided into quadrants by a black cross. These anisotropic sphero-crystals are apparently present in that state of matter which O. Lehmann [1904] designated as the "fluid crystalline phase". The rotation of the plane of polarised light by suspended particles as described above seems to be a property of fluid sphero-crystals, and Rosenheim and Tebb have called the phenomenon sphero-rotation. The cerebrosides and sphingomyelin of protagon tend to form mixed liquid crystals, a fact which explains to a great extent the difficulties encountered in their separation.

Short Historical Account of Protagon.

Though the name protagon was first used in 1865, there is ample evidence that substances similar to this body had already been isolated by former observers. Liebreich [1865] obtained protagon by treating brain with water and ether; on standing for some time the ether formed a separate layer and was removed. This treatment with ether was repeated, and after removal by filtration of as much water and ether as possible, the residue was extracted with 85 per cent. alcohol at 45°. The extract was allowed to stand at 0° when a precipitate formed. This precipitate was washed with ether, then moistened with water and purified by dissolving in alcohol at 45°. White crystals of protagon were thus obtained, their exact form being determined by the amount of alcohol used.

In spite of the fact that similar substances had previously been prepared and described by various investigators, this product of Liebreich, for some obscure reason, received a quite undue amount of attention. Consideration of the methods of preparation, and of the analytical data and properties of certain of these earlier bodies, leaves little doubt that the first substance of the nature of Liebreich's protagon was isolated by Fourcroy [1793], who obtained a "yellowish white matter" in the form of glistening scales on extracting brain with boiling alcohol. Vauquelin's [1812] "white matter," Couerbe's [1834] "cerebrote," Fremy's [1841; 1841, 1, 2] "cerebric acid," Gobley's [1847, 1850] "cerebrin," Von Bibra's [1854] "cerebric acid" and products isolated by a few other investigators were all of the same general nature as protagon. The "white matter" of Vauquelin differed in its method of preparation from protagon in that it had not been washed with ether, but Couerbe's "cerebrote" was at least in some cases prepared by a method practically identical with that of Liebreich.

The similarity of these substances to protagon is shown in the figures representing their composition. With the exception of the phosphorus content the agreement is wonderfully close, especially when we consider that the methods of analysis at the disposal of the earlier observers were very unreliable.

	C.	H.	N.	P.	S.[1]
Couerbe's cerebrote (1834)	67·82	11·10	3·40	2·33	2·14
Fremy's cerebric acid (1841)	66·7	10·6	2·3	0·9	—
Gobley's cerebrin (1850)	66·85	10·82	2·29	0·43	—
Bibra's cerebric acid (1854)	66·78	10·65	2·51	0·52	—
Liebreich's protagon (1865)	66·74	11·74	2·80	1·23	—

Liebreich paid but little attention to any of these earlier results, and dismissed all previous substances similar to protagon as "decomposition products". The alleged important point in Liebreich's preparation of protagon was the employment of alcohol at 45° for extraction of the brain substance. He declared that alcohol at a higher temperature decomposed protagon, and since previous investigators had made use of boiling alcohol, it was obvious that their substances could not have been protagon but a mixture of the products of protagon decomposition. Even boiling alcohol, however, does not decompose protagon [Thudichum, 1896; Gamgee, 1880], and it still remains somewhat of a mystery why Liebreich gave yet another unnecessary name to a substance which had already received so much attention in this respect. Liebreich himself believed that his protagon constituted the mother substance of practically all phosphorised bodies hitherto described, such as lecithin and cerebric acid. Hoppe Seyler [1866, 1], in

[1] All contained sulphur probably.

whose laboratory Liebreich worked, at first supported this view, and so for a few years protagon was hailed as one of the few known important substances essential for the carrying on of the vital processes in all animal and vegetable cells [Fischer, 1865, 1868; Hermann, 1866; Tolmatscheff, 1867].

Very soon, however, evidence began to accumulate which cast doubts on the claim that lecithin and similar substances were derivatives of protagon. Parke [1867] showed that egg yolk contained a phosphorised organic compound which was not protagon, while Hoppe Seyler [1866, 1867] stated that egg yolk and red blood corpuscles both contained lecithin, but were entirely free from protagon. Since the methods used in these investigations could not have decomposed any hypothetical pre-existent protagon, it gradually became clear that protagon did not stand to lecithin in the relationship of "mother substance" as claimed by Liebreich. Similar conclusions were arrived at by Diaconow [1867, 1867, 1].

A further blow was dealt at this product by Diaconow [1868, 1] who claimed to have obtained evidence that brain protagon was not a chemical unit. He considered that protagon consisted of a mixture of lecithin and some phosphorus-free fatty matter similar to a substance (cerebrin) isolated by Müller [1858]. Diaconow's results also strongly supported the view that lecithin existed preformed in the brain.

It is interesting to note that both Liebreich and Diaconow were pupils of Hoppe Seyler and obtained their divergent results while working in the same laboratory. Though Hoppe Seyler at first appeared to accept Liebreich's conclusions as to protagon without reserve, the results of Diaconow and other investigators, coupled with his own observations on red blood corpuscles, caused him to modify his previous views. Diaconow's contention that protagon was a mixture now received the weighty support of Hoppe Seyler, and various publications from his laboratory supported this view [Jüdell, 1869; Hoppe Seyler, 1869, 1871; Miescher, 1871].

Thudichum [1874], working independently, arrived at similar conclusions as to the non-unit nature of protagon, but did not agree with Diaconow that it consisted of lecithin and cerebrin. According to Thudichum, protagon was a mixture of cerebrin, phrenosin, kerasin and similar products together with myelin, kephalin and cholesterin. The view that protagon was a mixture was also supported by Petrowsky [1873], Bourgoin [1874] and by Gorup Besanez [1878].

In the light of all this evidence, there was at this period a general agreement among physiological chemists that protagon—whatever the nature of its constituents might be—was nothing more than a mixture of different substances. The claims of Liebreich were acknowledged to be based on faulty observation, and the protagon controversy seemed to have reached a final settlement. This, however, proved far from being the case, and protagon which seemed to be effectively buried in the early seventies has been resurrected subsequently on more than one occasion. The second important period in the history of protagon began in 1879, when Gamgee and Blankenhorn [1879] opened the closed protagon question and presented data indicating that Diaconow's conclusions were based on insecure foundations. Further, these observers claimed to have completely confirmed Liebreich's results. In this connexion some further reference to Diaconow's work may render matters somewhat clearer.

There seems little doubt that Diaconow's observation that protagon was a mixture from which lecithin and cerebrin could be obtained was the chief factor in bringing discredit on Liebreich's statements. That Diaconow's protagon was a mixture is true, but it is highly probable that his substance was a different body from Liebreich's protagon. In fact it would seem that Diaconow's protagon was really a mixture of lecithin (and kephalin) with Liebreich's protagon. Diaconow's method of preparation was in general similar to that employed by Liebreich—extraction of brain with alcohol at 40° and cooling the alcoholic extract to 0°, whereby a precipitate of impure protagon was obtained. As pointed out by Diaconow this crude protagon would naturally bring down some lecithin with it in the course of precipitation. It is also clear that the nature of the precipitate formed must have been dependent to some extent on the concentration of the alcoholic solution, since lecithin is easily precipitated from alcoholic solutions at 0° provided they are not too dilute.[1]

Therefore in the experiments of both Diaconow and Liebreich it is obvious that the primary precipitates obtained on cooling the warm alcoholic solution must have contained some lecithin. The final state of this precipitate as regards its lecithin content would depend on the thoroughness with which it was subsequently washed with ether. That Diaconow's substance was insufficiently washed is obvious from his own statement. His original precipitate obtained from alcohol at

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[1] This statement applies to kephalin even more so than to lecithin, and it is probable that the greater part of Diaconow's "lecithin" was really kephalin. The latter substance, however, was unknown at the time, and was included in the term lecithin. The word lecithin is here used in Diaconow's sense.

0° was filtered, washed with a *little cold alcohol* and then treated *once* with ether. Part dissolved in ether while part—protagon—remained. *On further extracting the protagon several times with ether, lecithin was found in the ethereal extract.* The amount of lecithin so found was obviously small, since its identification was carried out with very meagre amounts of material. Diaconow nevertheless attributed the whole of the phosphorus of protagon to the lecithin present. After separation of the lecithin by ether as described, the remaining ether-insoluble part of the protagon should have been lecithin-free and, therefore, on Diaconow's assumption phosphorus-free also. Diaconow, however, failed to obtain a phosphorus-free residue by ether extraction. Seeing that lecithin is exceedingly soluble in ether, the persistent presence of phosphorus in the ether-extracted residue ought to have suggested to this observer that all the phosphorus could not have been present as uncombined lecithin. Since a small part of the phosphorus was present as lecithin, Diaconow evidently *assumed*, without any justification, that lecithin accounted for all the phosphorus, and that a phosphorus-free body was also present—the cerebrin of Müller [1858]. In support of these assumptions, he produced no evidence whatever, and it is now known that his deductions were to a great extent fallacious, since the phosphorus of protagon is really accounted for by the presence of sphingomyelin. His statement as to the presence in protagon of a substance of the nature of cerebrin was based on an experiment in which a phosphorus-free residue was obtained from protagon *after boiling with baryta water.*

It is surprising that Diaconow did not suspect that his protagon was probably different from that of Liebreich in that it contained lecithin. It is also difficult to understand how he could have assumed that all the phosphorus of protagon was due to lecithin present in the free form. Assuming that the phosphorus was actually present as lecithin, all the evidence pointed strongly to the conclusion that much of this lecithin must have been combined in some form in which it was insoluble in ether. If so, it became necessary to accept Liebreich's conclusion that protagon was the mother substance of lecithin. This conclusion, however, was just what Diaconow's work most strongly controverted. On the whole an examination of Diaconow's investigations on protagon prove conclusively that he failed entirely to produce any direct evidence that protagon was a mixture, or that Liebreich's claims regarding the chemical unity of this substance were not fully justified.

Some of these points were considered by Gamgee and Blankenhorn [1879], who showed that Diaconow's criticisms of protagon were entirely valueless. These observers prepared protagon from dog and horse brains according to Liebreich's directions. Since the protagon obtained was exactly like the body described by Liebreich, and retained its composition when recrystallised four or five times out of alcohol, Gamgee and Blankenhorn concluded that protagon was undoubtedly a chemical unit. Similar results were obtained with protagon prepared by a modification of Liebreich's process, in which the brain, instead of being first treated with water and ether, was *directly* extracted with warm alcohol.

The subsequent development of the protagon story is a peculiar one. Gamgee and Blankenhorn's conclusions were accepted without demur by all chemists interested in the subject. Those who previously had firmly believed that Diaconow and his colleagues had finally disposed of protagon were now apparently as strongly convinced that they had been in error. The validity of Gamgee and Blankenhorn's alleged proofs of the unit nature of protagon was not questioned. With a wonderful unanimity Liebreich's conclusions were now accepted with as much readiness as they had formerly been rejected. This complete *volte face* was apparently justified by the results of researches which continued to be published for the next twenty years. Paper after paper appeared in confirmation of Gamgee and Blankenhorn's conclusions, and protagon seemed for a second time to be more strongly established than ever [Parcus, 1881; Baumstark, 1885; Kossel and Freytag, 1893; Ruppel, 1895; Zuelzer, 1899; Noll, 1899; Ulpiani and Lelli, 1902].

The results of some of these investigations actually indicated that protagon was not a chemical unit, though their authors failed to interpret them in this light. Thus Kossel and Freytag [1893] observed that certain protagons isolated by them failed to agree in composition with those obtained by the older researchers such as Liebreich and Gamgee. Since great attention was paid to the isolation of these substances, this difference in composition could not be ascribed to insufficient care in their preparation. In this connexion Kossel and Freytag point out that even in Liebreich's most carefully prepared protagon the figures for carbon and hydrogen in different specimens varied from 66·2 to 67·4 per cent. for carbon and 11·1 to 12·6 per cent. for hydrogen. Such results pointed definitely to the simple conclusion that protagon was not a chemical unit but a variable mixture. Kossel and Freytag, however, refused to accept the obvious interpretation of their results, but explained them by assuming the *existence of different protagons*. The problem had become more complicated than ever.

One investigator alone during all these years steadfastly refused to come into line and accept the orthodox views. Year after year, Thudichum [1874-1896] produced evidence, based on careful and extensive researches, that protagon was after all but a mixture of substances which could be separated to a great extent by repeated recrystallisation from alcohol.

With ever-increasing bitterness he assailed everybody who refused to accept his own conclusion that protagon had no claims to be considered as a chemical unit. In spite of the extreme if somewhat misplaced vigour with which Thudichum conducted his campaign, no impression whatever was produced on his opponents. For more than twenty years, Thudichum was but a voice crying in the wilderness, and protagon was still firmly believed in. It is probable that the small amount of attention bestowed on Thudichum's work, both here and in other connexions, was in a great measure due to his unfortunate use of personal methods of attack in controversies which should have been entirely impersonal. Thudichum's persistence, however, seemed at last to bear fruit, and his conclusions received support from an observation made by Wörner and Thierfelder [1900] that on treatment of protagon with a mixture of alcohol and benzene a phosphorus-free body was obtained. This body they called *cerebron*. The residue left after the separation of cerebron had the appearance of ordinary protagon, yet all former protagons must have contained this substance. Another phosphorus-free body was also obtained. These results indicated that protagon was undoubtedly a mixture, a conclusion which was further supported by certain variations found in the analytical figures of some of Thierfelder's specimens.

Koch [1902] treated protagon with glacial acetic acid two or three times, and isolated a substance free from phosphorus which he took to be cerebrin. He considered that protagon was a mixture of lecithin, kephalin and cerebrin. Lesem and Gies [1903] also advanced evidence that protagon was not a unit substance. By 1904 it appeared to be generally accepted that this much-discussed body was undoubtedly a mixture [Koch, 1904; Hammarsten, 1904].

Although protagon was now buried for the second time, it did not enjoy a long repose. Another epoch in its history began in 1904, when a publication by Cramer [1904] appeared claiming to have once more established the chemical unity of the substance. This paper formed the starting-point for a renewal of the old discussion as to the nature of protagon, the principal protagonists being Cramer, who upheld Liebreich's contentions, and Rosenheim and Tebb, who, along with Gies and his collaborators, refused to believe in protagon. The discussion was carried on with a good deal of the vigour which characterised the older controversies, and which seems to be evoked by investigations on this particular substance.

It is impossible in this short monograph to give more than a brief general outline of the arguments advanced by these two schools for and against the unit nature of protagon. It would appear, however, that some of the later statements of the Cramer school are not quite consistent with evidence brought forward by the same school at an earlier period. Indeed the impression produced is that the unit nature of protagon had to be upheld at all costs, and though a good deal of ingenuity was exercised in this endeavour, it appears to the author that the evidence is altogether in favour of Rosenheim and Tebb's conclusion that protagon is a mixture of cerebrosides and phosphatides.

Cramer contended that the reasons brought forward by Wörner and Thierfelder [1900] and by Lesem and Gies [1903] against the chemical individuality of protagon were not conclusive. He claimed that the protagon investigated by these observers was impure,[1] and that the cerebron which Thierfelder obtained from protagon was identical with a substance previously isolated by Gamgee [1880] from brain-tissue and called by him pseudo-cerebrin (p. 118). This body was extracted from brain by alcohol at 45°, and so would naturally be present in protagon provided no special steps were taken to ensure its removal. Cramer maintained that the protagon of Gamgee was a pure substance and based much of his defence of protagon on this assumption.

According to Gamgee, pseudo-cerebrin, being less soluble in 80 per cent. alcohol at 45° than protagon, could be separated from crude protagon by "repeated recrystallisation" from 80 per cent. alcohol. By this procedure Gamgee obtained residues of pseudo-cerebrin from "impure protagon". In order, therefore, to obtain protagon free from pseudo-cerebrin *repeated* recrystallisation was necessary [Gamgee, 1880]. In the light of these statements it is by no means clear how

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[1] Cramer [1908] afterwards admitted that the protagons of Lesem and Gies were pure ["Quart. Journ. of Exper. Physiol.," 1, 102].

Gamgee and Blankenhorn were able to obtain pure protagon after only *two* recrystallisations from alcohol as they claim to have done.

In this connexion it is interesting to note that Gamgee and Blankenhorn's paper, indicating that protagon was a unit chemical body, was published in Hoppe Seyler's "Zeitschrift" in 1879. At this time Gamgee was obviously unaware of the existence of pseudo-cerebrin. Shortly afterwards he succeeded in separating this body, but instead of communicating his result to the scientific world through the usual channels, he was content to restrict all mention of his discovery to a short account which appeared in his text-book in the following year (1880).

From the properties of pseudo-cerebrin, it must have been obvious to Gamgee that many of the older protagons contained this body. Indeed one is tempted to ask whether, after discovering pseudo-cerebrin, Gamgee had not of necessity the suspicion forced upon him that the protagons of Gamgee and Blankenhorn [1879] also contained pseudo-cerebrin and were therefore mixtures. Whether or not this was so is unknown, for he published no more on the subject. A consideration of Gamgee's description of the properties of pseudo-cerebrin, taken in conjunction with the account of the methods of preparation of protagon furnished by Gamgee and Blankenhorn, leave no room for doubt that all their alleged pure protagons contained pseudo-cerebrin. Indeed, it is certain that all protagons must have contained this substance, so that Cramer's observations regarding pseudo-cerebrin rather increased than decreased the probability that protagon was a mixture of pseudo-cerebrin (cerebron, cerebrin) with other substances.

Another point advanced by Cramer [1904] appeared at first sight to be of more importance. Thudichum accounted for the general similarity of composition of different protagons by the fact that they were all prepared in the same way, and Cramer argued that if protagon was a mixture, a different method of preparation should yield a different substance. Cramer therefore treated minced brain with a hot solution of sodium sulphate, and then heated the mixture in a water-bath at 100° till the brain substance coagulated. On getting rid of as much as possible of the sodium sulphate solution, the residue was extracted once with 95 per cent. alcohol, and afterwards with 80 per cent. alcohol till no more substance separated on cooling. Further purification was carried out on the usual lines. By this method Cramer obtained a substance similar in composition and properties to the protagon of the older observers. He argued that this result supported his contention that protagon was a chemical unit. On examination, however, it does not appear that Cramer's method of preparing protagon differed *in any essential way* from that of the older observers. As pointed out by Posner and Gies [1905] this preliminary heating does not affect the chemical composition of protagon, its main effect being to coagulate the brain proteins. Thus, no great difference in composition of the resulting product was to be expected. Cramer's protagon, like all other protagons, contained pseudo-cerebrin [Posner and Gies, 1905, 1].

Like other investigators, Cramer found that one of his specimens of protagon, prepared in the same way as the others, differed considerably in composition from average protagon. Following Kossel, he assumed that this substance represented one of a group of different protagons, and suggested that the members of this alleged group should be called homoprotagons to distinguish them from ordinary protagon. Thus, another indication of the non-unit nature of protagon was obscured by the introduction of an unnecessary name. The assumption of the existence of homo-protagons led directly to the idea held by the Cramer school that protagon may be a mixture of *homologous protagons*, a view advanced by Fränkel [1909].

Cramer's claims were thoroughly examined by Posner and Gies [1905 ; 1905, 1] who, as the result of a long and careful investigation, brought forward evidence that should have ended the controversy. They showed that protagon was a mixture of substances in which a phosphorus-free body or bodies were associated with one or more substances containing relatively large proportions of phosphorus : the exact nature of these components was not known. In confirmation of the results of Thudichum [1896] and of Lesem and Gies [1903] they showed that " by fractionation at the same or different temperatures in 85 per cent. alcohol, under conditions that could not effect chemical decomposition, protagon was partitioned into products very dissimilar in phosphorus—and sulphur—contents ". They also found that Cramer's method yielded typical protagon " which could be fractioned without chemical decomposition into products of variable phosphorus content similar to those obtained from protagon prepared by other methods ".

Some of the results in phosphorus percentage obtained by Posner and Gies [1905, 1] from protagon many times recrystallised from 80 per cent. alcohol at 45° and cooled to 0° are shown in the table. It is important to note that the product obtained during the processes of recrystallisation was not dried before being dissolved for the next crystallisation.

No. of Times Recrystallised.	Percentage in Crystals.	Percentage in Mother Liquor.
1	1·22	—
2	—	—
3	—	—
4	1·21	—
5	—	—
6	—	1·42
7	—	—
8	—	1·05
9	—	0·96
10	0·93	1·02

The product obtained on the tenth recrystallisation was dried and subjected to "fractional crystallisation"[1] by treatment with 85 per cent. alcohol at 40° for fourteen hours. By allowing the solution to stand at different temperatures (28° and – 3°), precipitates and filtrate residues with the following percentages of phosphorus, were obtained:—

0·95, 1·22, 0·89, 1·12, 0·74, 0·98, 0·74, 1·68, 1·31, 0·47.

Lochhead and Cramer [1907] refused to accept the conclusions which Posner and Gies deduced from these figures, and argued that strong evidence of the unit nature of protagon was afforded by the fact that after ten times repeated crystallisation from alcohol the crystals of protagon separating out had the same phosphorus percentage as the residue in the mother liquor,[2] and incidentally agreed in this respect with Gamgee's protagon. The results of the *fractional* crystallisation of the purified protagon, Cramer [Wilson and Cramer, 1908] accounted for by the assumption that treatment with alcohol at 40° for fourteen hours decomposes protagon. As the Cramer school persistently quote the first portion of this experiment as proof of their own contention that protagon is a unit chemical substance, it is necessary to examine their claims somewhat more fully.

From the descriptive details of this experiment furnished by Posner and Gies, it is certain that the protagon obtained on the tenth recrystallisation was already subjected *for more than twenty-four hours to*

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[1] Fractional crystallisation consisted in dissolving the substance in a warm solvent and filtering off the fractions which separated at different temperatures as the solution cooled.

[2] See table above.

the influence of 85 *per cent. alcohol at* 45°.[1] According to Wilson and Cramer this product consisted of pure protagon. How then could a substance which had withstood the action of hot alcohol (45°) for more than twenty-four hours be decomposed by alcohol at a lower temperature (40°) in fourteen hours? Cramer's contentions demand that it should be so.

On careful examination it appears that one important difference between the ordinary crystallisations used for purifying protagon and the subsequent "fractional crystallisation" is that, in the former case, the protagon separating from alcohol on each recrystallisation was dissolved in fresh alcohol *without being previously dried*, while in the process of fractional crystallisation, the protagon *was dried* before being dissolved in alcohol. This experiment therefore suggests that dry protagon differs from moist protagon in its behaviour towards alcohol, and might even indicate that moist (hydrated?) protagon which had never been dried, is really composed of substances present in some form of very feeble combination which is destroyed on drying.

That moist protagon can be fractionated as easily as dry protagon is evident from an experiment of Rosenheim and Tebb [1909], in which it was shown that protagon obtained by Cramer's method was easily divided into fractions containing varying percentages of phosphorus. This sample of protagon *had not been dried during the processes of recrystallisation*, and yet under conditions which, according to Cramer, do not give rise to decomposition, the various fractions gave the results indicated below.

	Phosphorus Percentage.
Original "pure" protagon	0·98
1st Recrystallisation	0·66
5th "	0·27
7th "	0·13
Fraction from mother liquor	2·54

Further, Gies [1907] pointed out that the figures[2] of Posner and

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[1] Though elaborate details of this experiment are furnished by Posner and Gies, it is, unfortunately, somewhat difficult to ascertain from their description the exact number of hours during which the protagon was subjected to the action of alcohol at 45°—the temperature at which solution was effected. In the process of dissolving the protagon for *one* of the *ten* crystallisations carried out, it is definitely stated that extraction was "continued for twenty-four hours". From this it is obvious that, altogether, the protagon must have been treated with hot alcohol for very much more than twenty-four hours; indeed, it is probable that the treatment lasted for 100 hours or more.

[2] See table, p. 134.

Gies were really of no significance in the sense advocated by Cramer, since the physical qualities of the precipitates from alcohol, and the residues obtained from the corresponding filtrates showed that they were not identical substances. He further argued that the slight difference in the phosphorus percentage[1] of these substances was of sufficient importance to suggest of itself that they were not identical products. Thus, while the fact that protagon which had been recrystallised ten times from alcohol had the same percentage of phosphorus as the mother liquor, at first sight suggests that this product *might be* a chemical unit, it offers *per se* no *proof* whatever that this is so, and could only be advanced in support of this contention if other supporting evidence were forthcoming. Such evidence as we have, all points to the opposite conclusion—that protagon is a mixture.

The observations of Posner and Gies were confirmed by Rosenheim and Tebb [1907], who by means of somewhat different methods arrived at similar conclusions. These observers strongly emphasised the fact that by fractional crystallisation with acetone and other solvents, protagon could be split up into substances of varying nitrogen and phosphorus content. These conclusions of Rosenheim and of Gies were criticised by Wilson and Cramer [1908; 1908, 1], who maintained that many of those results had no bearing on the question at issue, since, in their opinion, they were obtained from material *called* protagon, which was not identical with protagon, but represented a crude product containing protagon together with other substances. They admitted that there was evidence, based chiefly on the work of Gies and his collaborators, that pure protagon when subjected to a "process of *fractional* crystallisation" could be split into substances differing widely in their solubilities and in their phosphorus content.

From the mass of experimental work carried out on protagon it was now accepted by all investigators that this body consisted chiefly of two classes of substances, one of which was poor in phosphorus (cerebrosides) while the other was comparatively phosphorus-rich (the phosphatide, sphingomyelin).

The admission by Wilson and Cramer that protagon could be divided into different substances by "fractional crystallisation" demanded, from their point of view, the obvious supposition that during this process decomposition had taken place. Since fractional crystallisation as used by Posner and Gies consisted in the treatment of protagon with a quantity of warm alcohol over periods lasting many hours, it was necessary to show that the prolonged action of warm alcohol on protagon resulted in decomposition. A sample of protagon was subjected to the action of 80 per cent. alcohol at 44° for twenty-two hours and then compared with a similar sample which had not undergone this treatment. The results showed that definite changes were produced in the specific rotation and refractive index of protagon so treated, and from this Wilson and Cramer argued that warm alcohol decomposed protagon. Some of the figures which they obtained were as follows. The protagon investigated was dissolved in pyridine.

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[1] The estimation was carried out both by Posner and Gies and by an independent observer: in each case the percentage of phosphorus in the precipitate from alcohol was lower than that from the filtrate, so that the difference naturally indicated something more than experimental error.

	Specific Rotation.	Refractive Index of 3 Per Cent. Solution at 30°.
(1) Protagon (before alcohol treatment)	6·66	1·5034
(2) Protagon (after alcohol treatment at 44° for twenty-two hours) . . .	13·08	1·5038

From these figures it is clear that prolonged action of warm alcohol does produce a marked change in the specific rotation of the substance.[1] There is no reason to ascribe this to "decomposition".

Such a change tells with equal force for the presence of a mixture of optically active substances, one or more constituents of which become racemised by heating, and cannot be considered as evidence in support of the chemical unity of protagon [Rosenheim and Tebb, 1909].

Another argument used by the Cramer school in support of their claims for protagon, was the fact that samples of protagon obtained by various investigators possessed a remarkably constant chemical composition, as indicated by elementary analyses. This similarity is well indicated in the figures quoted by Wilson and Cramer and given below:—

Investigator.	C. Percentage.	H. Percentage.	N. Percentage.	P. Percentage.	S. Percentage.
Gamgee . .	66·39	10·69	2·39	1·06	—
Baumstark .	66·48	11·12	2·35	1·02	—
Ruppel . .	66·29	10·75	2·32	1·13	0·096
Lesem and Gies	66·11	10·90	2·02	1·23	0·77
Cramer . .	66·37	10·82	2·29	1·04	0·71

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[1] Rosenheim and Tebb [1908, 1] could not obtain this result.

Similar as are these results, it is obvious that *per se* they supply no proof of the unit nature of protagon. No doubt, *when considered quite apart from other evidence*, they suggest that protagon is not a mixture. Though Rosenheim has pointed out that a review of all the analyses of protagon in the literature furnish figures which are often by no means in such close agreement as those given above, it must be admitted that the analytical data obtained from different protagons agree, on the whole, remarkably well. This agreement is a superficial one, and is only apparent in products prepared under certain given conditions and recrystallised from one solvent (alcohol) under equally restricted conditions [Rosenheim and Tebb, 1909]. It is dependent on the fact that the substances of which protagon is composed have somewhat similar solubilities in cold alcohol, and so cannot be readily separated under the conditions usually observed in the preparation of protagon. A similar phenomenon holds in the case of lecithin, which, though a mixture of two substances, cannot be separated by solvents into its components, but remains of approximately the same composition as the original lecithin [MacLean, 1915]. Another example is seen in the case of phytosterin, which on recrystallisation from alcohol, usually furnishes a product of constant composition having the same chemical and physical properties. This substance, ever since its discovery by Hesse [1878], was assumed to be a chemical unit, until Windaus and Hauth [1906] demonstrated that it was a mixture of two substances. Margaric acid may also be referred to as a product of this nature [Holde, 1905].

It therefore follows, that the recrystallisation of a substance from alcohol without change in its composition, is no proof of its chemical entity. On the other hand, if "pure" protagon could be separated into fractions differing in chemical composition, by recrystallisation from alcohol or other solvents, and under conditions excluding the possibility of decomposition,, it must be a mixture. Such evidence would be incontrovertible in establishing the nature of protagon, for, unlike many of the results brought forward in favour of the unit nature of this body, this evidence would bear only one possible interpretation. The results of recrystallisation of protagon from solvents under suitable conditions must therefore be accepted as the crucial test, for or against the view, that protagon is a chemical entity.

In view of the alleged effect on protagon of prolonged treatment with hot alcohol, Wilson and Cramer [1908] proposed a method for the preparation of protagon in which they used boiling alcohol, but restricted its use to a very short space of time (one to two minutes). These observers stated that the substance prepared in this way was pure protagon, and that the only conditions necessary for recrystallisation from alcohol, without change in its composition, was exposure to the hot solvent for a short period. These claims were examined by Rosenheim and Tebb [1908], who prepared protagon according to Wilson and Cramer's instructions. They found that by simply varying the proportion of protagon to alcohol, variations in the phosphorus percentage of over 50 per cent. were obtained; on the other hand, as might be expected, little or no change in the phosphorus content was observed when the amount of alcohol used was small.

The following figures are taken from one of their experiments in which protagon was recrystallised from alcohol. The exposure of the protagon to boiling alcohol in the process of recrystallisation was strictly limited to one minute according to Cramer's directions. It will be seen that the phosphorus percentage fell from 1·33 to 0·39.

	Proportion of Protagon to Alcohol.	Phosphorus Percentage.
Original protagon	—	1·33
1st recrystallisation	1 : 18	0·83
2nd ,, ...	1 : 30	0·54
3rd ,, ...	1 : 20	0·44
4th ,, ...	1 : 20	0·39

Another experiment shows the result when small amounts of alcohol were used.

	Proportion of Protagon to Alcohol.	Phosphorus Percentage.
Original protagon	—	1·18
1st recrystallisation	1 : 5	0·99
2nd ,, ...	1 : 2	0·96

Even more striking evidence that protagon is a mixture was obtained when pyridine was used as a solvent. Protagon is fairly soluble in pyridine at 30 to 45° and a precipitate forms on cooling. This precipitate is very different from the original protagon, as the appended figures show.

	Phosphorus Percentage.	Nitrogen Percentage.
Original protagon	1·07	2·46
Fraction I (Insoluble in pyridine at plus 15°)	2·51	3·03
Fraction II (Precipitated by acetone at 0°)	0·09	1·73

This evidence of the non-unit nature of protagon is as complete as can reasonably be required, but it was further strengthened by results obtained from protagon, when a variety of different solvents and mixtures of solvents were employed [Rosenheim and Tebb, 1909, 1910]. The extraordinary variation in the phosphorus percentage of fractions obtained from so-called pure protagon is very remarkable, as may be seen from the following experiments showing variations from 0·05 per cent. to 2·6 per cent. obtained in a single recrystallisation of a protagon containing 0·99 per cent. phosphorus.

Solvent.	Proportion of Mixed Solvents.	P. Percentage in Original Protagon.	P. Percentage in Recrystallised Substance.
Trichlorethylene	—	0·99	0·70
,, plus alcohol	1 : 4	—	0·48
,, plus acetic acid	1 : 2	—	0·05
,, ,, ,,	1 : 3	—	0·36
Chloroform plus acetone	1 : 3	—	0·83
,, ,, acetic acid	1 : 2	—	0·08
,, ,, alcohol	1 : 2	—	0·60
Pyridine	—	—	2·60

Another protagon containing 1·08 per cent. of phosphorus gave equally striking results after one recrystallisation from different solvents.

Solvent.	Proportion of Mixed Solvents.	P. Percentage in Original Protagon.	P. Percentage in Recrystallised Substance.
Carbon tetrachloride plus alcohol	1 : 4	1·08	0·53
,, ,, ,, acetone	1 : 1	—	0·71
Chloroform plus alcohol	1 : 2	—	0·25
,, ,, acetone	1 : 1	—	0·79
,, ,, acetic acid	1 : 2	—	0·16
Trichlorethylene plus alcohol	1 : 4	—	0·41
,, ,, acetone	1 : 2	—	1·01
Pyridine	—	—	2·46

Rosenheim and Tebb [1908, 2] also showed that an artificial mixture of cerebrosides and sphingomyelin, in the proportions in which they occur in protagon, had the same composition and properties as protagon, and could be recrystallised from alcohol without change.

Further, the same observers demonstrated that the different lipins of brain could be separated by consecutive extractions with different solvents. This method of selective extraction gave the results indicated, the dried brain being treated with the different solvents in the order given [Rosenheim and Tebb, 1910, 1].

Solvent. *Lipin Extracted.*
1. Cold acetone = Cholesterol.
2. Ether = Lecithin and Kephalin.
3. Pyridine (30 to 40°) = Cerebrosides.
4. Pyridine (45°) = Sphingomyelin.

In this experiment the highest temperature employed was 45° so that decomposition was practically excluded, yet the constituents of alleged protagon were extracted in separate fractions. The results show that cerebrosides and sphingomyelin exist in the free state in the brain, and this view must be accepted. It is true that the Cramer school [Pearson, 1914] attributes these results to a decomposing action of pyridine on the hypothetical protagon present in the brain. This explanation is rather interesting, in view of the fact that Cramer's physical constants of protagon were obtained from solutions of this product in pyridine. If pyridine decomposes protagon it must surely be admitted that no importance can be attached to these constants, yet Cramer bases his statement that protagon is decomposed by warm alcohol entirely on results obtained from solutions of protagon in warm (30°) pyridine.[1]

These results appear to furnish conclusive evidence that protagon is a mixture, for it is impossible to interpret them in any other light. Cramer [1910] admitted the correctness of Rosenheim's observations in the recrystallisation experiments described, but argued that they were not valid, since the protagon of the latter was not true protagon but a mixture. It is, therefore, obvious that the question had now arrived at a stage at which the answer entirely depended on the identity of the specimens of protagon used by Cramer and Rosenheim respectively. In order to demonstrate conclusively that these protagons were similar substances, Rosenheim endeavoured to procure a specimen of Cramer's protagon for comparison with his own. Unfortunately, Cramer was unable to supply such a specimen, and so no direct comparative experiments could be carried out. It is, however, impossible to accept the claim that Rosenheim's specimens differed from those of Cramer, as many of them were obtained by scrupulously following Cramer's directions; if they did differ in any way, this affords further proof of the non-unit nature of protagon, for it is certain that any unit substance must have yielded exactly similar products to both observers, since their methods of preparation, in many cases, were absolutely identical. Even if, for purposes of argument, Cramer's contention that Rosenheim's preparations were impure be allowed, this does not materially elucidate matters, for in order to obtain Rosenheim's results, the amounts of impurity present must have been enormous; this possibility is quite excluded by the method of isolation adopted by Rosenheim.[1]

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[1] It might well perhaps be argued that pyridine at 30 to 40° (Rosenheim) decomposes protagon, while pyridine at 30° (the temperature adopted by Cramer) does not do so. Though this is improbable it may be pointed out that Rosenheim and Tebb [1908, 1, 3] have proved that protagon can be fractionated by pyridine at exactly 30°. The only obvious answer to this would be that such a protagon was, from Cramer's standpoint, an impure product. Such an explanation is inadmissible, since the protagon in question was obtained by Wilson and Cramer's method.

In spite of all this evidence that protagon is a mixture, Pearson [1914] has made still another endeavour to rehabilitate the substance. Pearson estimated the effect of protagon on the boiling-point of a liquid, and compared the result with the effect produced by the individual constituents of protagon (phrenosin, kerasin and sphingomyelin). Since the molecular weight of each of these three bodies probably lies somewhere in the region of 1000, Pearson argued that if they followed Raoult's law, solutions of moderate concentrations of these substances should produce an appreciable effect in raising the boiling-point of a solvent such as chloroform. On the other hand, protagon, if it were merely a mixture, should give the same result as a mixture of its components, while, if it were a definite chemical unit, its molecular weight would be so high (at least 3000) that but little, if any, effect on the boiling-point could be expected.

Pearson claims to have demonstrated that protagon itself in moderate concentration produces no rise in the boiling-point of chloroform, while, if it be decomposed into its constituents, a mixture of these in chloroform solution produces a slight elevation of the boiling-point of the solvent. Rosenheim [1916] has already pointed out that the mixture of decomposition products obtained does not completely represent the original protagon. The elevations of boiling-point produced by the addition of the decomposition products are small and are irregular in character. Thus, in two of the experiments quoted, increasing concentration of the cerebrosides produced an elevation of boiling-point out of proportion to the amount of substance added, whilst in the last experiment recorded, increase of concentration resulted in a diminished rate of elevation. Much more attention must be paid to the purity of the substances, and to the elimination of impurities possibly introduced in the method of preparation of the decomposition products, before any significance can be attached to such results.

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[1] See also Cohen and Gies [1908].

Protagon is nothing more than a mixture of cerebrosides and sphingomyelin with traces of other bodies.

CHAPTER VI.

ALLEGED LIPINS.

Carnaubon.

DUNHAM [1905] showed that ox kidneys contained a substance similar to Liebreich's "protagon". On comparing the two products prepared by identical methods from brain and from kidneys (extraction of the fresh tissue with 85 per cent. alcohol [Liebreich, 1865] or with boiling alcohol after the coagulation of the proteins by 5 per cent. sodium sulphate solution [Cramer, 1904]), he found that the substance from the kidney contained more nitrogen and phosphorus than that from the brain, but from the nature of the cleavage products, he concluded that both substances belonged to the same group.

Later, Dunham [1908] succeeded in isolating from his kidney product an acid of the composition $C_{24}H_{48}O_2$, which he considered identical with carnaubic acid. As this fatty acid of vegetable origin had not been previously found in animal tissues, Dunham and Jacobson [1910] gave the name "carnaubon" to this protagon-like lipin of the kidney. From their hydrolysis experiments, these observers came to the conclusion that carnaubon represented a new type of lipin having as its basis, not glycerine as in lecithin, but amino-galactose, and that this amino sugar was combined with three fatty acids (carnaubic, stearic and palmitic acids) and with phosphoric acid, whilst the latter was further combined with two choline groups. The relationship of N : P in this body was 3 : 1.

MacLean [1912, 1] obtained a similar lipin from horse kidney, but found that his substance contained a water-soluble nitrogenous impurity. On the removal of this impurity, a product was obtained with a N : P ratio of exactly 2 : 1. This observer [1912, 2] pointed out the resemblance of this substance to the diamino-monophosphatide, sphingomyelin, and suggested that the very small amount of sugar which it still yielded on hydrolysis might be due to traces of impurities. At this point it was fairly obvious that "carnaubon" was probably a mixture of sphingomyelin with traces of cerebrosides. This view was substantiated by Rosenheim and MacLean [1915] who made the following observations:—

1. The acid $C_{24}H_{48}O_2$ present in "carnaubon," and called carnaubic acid by Dunham and Jacobson, was not carnaubic acid but lignoceric acid, an acid present in sphingomyelin and in the cerebroside kerasin. No palmitic or stearic acids could be found.
2. Another acid, phrenosinic acid ($C_{25}H_{50}O_3$), was isolated which is the acid present in the cerebroside phrenosin.
3. Besides choline, another base identical with sphingosine was found. This base is present in sphingomyelin and in the cerebrosides.

Since carnaubic acid is not present, there is obviously no justification for the further use of the term "carnaubon". Indeed, the existence of so-called "carnaubic acid" itself has been questioned [Röhmann, 1905; Matthes and Sanderr, 1908; Lewkowitsch, 1913].

Another interesting point in connexion with this kidney substance is the fact that its elementary composition, as found by different observers, is extremely variable. The amount of galactose split off on hydrolysis also differs very materially. Thus, in the samples examined by Dunham [1905], Dunham and Jacobson [1910] and MacLean [1912, 2] the following variations were found:—

C. Per Cent.	H. Per Cent.	N. Per Cent.	P. Per Cent.	S. Per Cent.	Galactose.
65·61 to 68·19	11 to 12·37	2·55 to 3·26	2·02 to 3·44	0 to 0·82	Traces to 13·78

In the light of all these facts, it is obvious that "carnaubon" is simply so-called "protagon," and, like the latter, represents a variable mixture of lipins mainly consisting of cerebrosides (phrenosin and kerasin) and sphingomyelin. The kidney "protagon" mixture seems to contain more sphingomyelin and less cerebrosides than the corresponding brain "protagon".

Paranucleo-protagon.

According to Ulpiani and Lelli [1902] protagon forms a compound with protein, which is present as such in the brain. This supposed combination was isolated by treating a chloroform extract of brain with ethyl acetate when a precipitate formed. To this precipitate Ulpiani and Lelli gave the name "paranucleo-protagon," since on prolonged treatment with 85 per cent. alcohol at 45° it was decomposed into protagon and paranuclein, the latter being obtained as an insoluble residue. From what has already been said about "protagon" it follows that paranucleo-protagon must have been a mixture of protagon and protein material. That paranucleo-protagon does not constitute a chemical unit has been shown by the investigations of Steel and Gies [1907] and Posner and Gies [1905].

Other Insufficiently Characterised Lipins.

While the evidence at our disposal at present points to the conclusion that all organs contain certain amounts of lecithin and kephalin, and probably sphingomyelin and cerebrosides as well, certain other lipins, differing from those mentioned, have been stated to be present in certain organs.

Many of these substances have been isolated and named by Fränkel and his collaborators, but it is no exaggeration to say that Fränkel has produced no reliable evidence whatever that any of his alleged new bodies exist. Indeed, it is certain, as pointed out below, that several of Fränkel's new products were mixtures of lecithin with other substances.

These new bodies were generally obtained in the form of the cadmium chloride combination, which was purified by precipitating its benzene solution with alcohol. From the behaviour of carnithin-cadmium chloride combinations, carnithin must have been present in practically all cases. The nitrogen : phosphorus ratio affords no reason for accepting the product as a definite lipin body. By precipitating extracts of organs with cadmium chloride according to Fränkel's procedure, products with almost any N : P ratio can be obtained.

Certain other lipin bodies have been isolated from time to time by various observers and especially by Thudichum. In Thudichum's methods for the preparation of lipins very drastic measures were often employed, so that some at least of his substances were, almost certainly, decomposition products.

It may be that future research will show that some of these ill-characterised bodies do really exist in the tissues, but, up to the present, the evidence is not such as to justify their inclusion among the well-defined lipin bodies. Many of them, on the other hand, can be shown to be mixtures. The principal bodies of this nature are as follows:—

Group I.—Monamino-monophosphatides like Lecithin.
N : P = 1 : 1.

1. Vesalthin.

[Fränkel and Pari, 1909.]

This name was given by Fränkel and Pari to a substance isolated by them from ox pancreas. The pancreas was freed from fat, dried at 100° for some hours, extracted with cold acetone and finally with hot acetone. On cooling the hot acetone extract a yellow mass was precipitated which was dissolved in hot 96 per cent. alcohol. This hot alcoholic solution, on cooling, also gave a precipitate consisting chiefly of fat. To the clear alcoholic filtrate, freed as much as possible from fat, excess of cadmium chloride was then added; the precipitate was dissolved in hot dry benzene from which, on cooling the solution, it was reprecipitated by the addition of absolute alcohol.

The cadmium chloride compound contained $C = 46{\cdot}76$, $H = 7{\cdot}78$, $N = 1{\cdot}73$, $P = 3{\cdot}87$, $Cd = 13{\cdot}79$, $Cl = 8{\cdot}71$ per cent., and corresponded to a compound of the empirical formula $C_{32}H_{63}NPO_9 . CdCl_2$. It was supposed to represent vesalthin cadmium chloride.

This substance had the same N : P ratio as lecithin, and it is quite certain, from the account of its preparation, that the final alcoholic solution from which the cadmium chloride compound was obtained must have contained lecithin. Lecithin is fairly soluble in hot acetone especially in the presence of other fatty bodies, and is of course easily soluble in alcohol. On cooling the hot acetone solution, much of the lecithin present would separate, to be taken up in the subsequent treatment with alcohol. When this alcoholic solution was finally treated with cadmium chloride, any lecithin present must have been precipitated. What other substances were mixed with the lecithin is not quite certain. It is probable that some lecithin partly decomposed by the original heating to 100° was present, or, as suggested by Bang, vesalthin might contain some "denaturated phosphatide". At any rate, vesalthin from its method of preparation could not have been a pure substance.

Fränkel and Pari claimed that vesalthin contained myristic acid ($C_{14}H_{28}O_2$) together with an acid of the oleic acid series but with a less number of carbon atoms: glycerophosphoric acid was also probably present along with a base which did not appear to be choline.

The statement that myristic acid was a constituent of vesalthin was based solely on the observed melting-point of an acid separated on hydrolysis. The presence of the other acid was apparently assumed, in order to account for the facts that vesalthin was unsaturated, and gave on analysis figures indicating a low carbon and hydrogen content. It is unnecessary to say more regarding these acids, for the melting-point alone cannot be accepted as of any value whatever in indicating the presence of a particular acid in a mixture of unknown acids.

The claim that the base present was not choline, was based chiefly on the observation that the product contained four methyl groups instead of three as in choline. It is now known, however, that the estimation of methyl groups in lipins by the method employed gives entirely fallacious results.

As a matter of fact, the platinum content of the platinum chloride combination of the base agreed with that of choline platinum chloride though the melting-point (249 to 254°) was somewhat too high [Fränkel, Linnert and Pari, 1909]. Choline platinum chloride may be made to show great variations in its melting-point according to the method of heating, and Trier [1912] found that samples of the pure crystalline product melted at temperatures varying from 230 to 257°.

There seems no doubt that vesalthin was a mixture [MacLean, 1914], and the claims put forward on its behalf have never been substantiated. The name should therefore be dropped.

Two other bodies belonging to this group are Thudichum's [1884] paramyelin and myelin. It is probable that paramyelin, at any rate, was a decomposition product: myelin has apparently more claims to be considered as a chemical unit, but the evidence is not sufficient for a definite statement on this point.

2. Acetone-soluble Vesalthin-like Body from Heart.

[Erlandsen, 1907.]

A body very similar to vesalthin was isolated by Erlandsen from ox heart: it was obtained as the platinum compound. The dried heart tissue was extracted with ether, the ethereal extract concentrated to a syrup and excess of acetone added; on separating the precipitated lecithin, the acetone solution was evaporated, the residue extracted with a little alcohol and excess of platinum chloride added to the alcoholic solution.

The precipitate thus obtained corresponded to the empirical formula $(C_{33}H_{62}O_8NP)_2H_2PtCl_6$.

N = 1·8 per cent.; P = 3·69 per cent.; N : P ratio = 1·09 : 1.

It will be seen that this product agreed fairly well in composition with vesalthin.

That Erlandsen's product was a mixture of lecithin and other bodies was shown by MacLean [1914], who prepared the body exactly according to Erlandsen's directions, and after purification, obtained from it ordinary lecithin.

Group 2.—Diamino-monophosphatides. N : P = 2 : 1.

Alleged substances of this nature have been isolated from ox heart [Erlandsen, 1907] and from kidney [Fränkel and Nogueira, 1909]. Both products were obtained as cadmium chloride compounds. MacLean [1914] showed that Erlandsen's substance was a mixture of lecithin contaminated with carnithin; the method of preparation employed by Fränkel suggests that Fränkel and Nogueira's product is a similar mixture. The only diamino-monophosphatide hitherto isolated from the tissues is sphingomyelin.

Group 3.—Triamino-monophosphatides. N : P = 3 : 1.

Neottin.

[Fränkel and Bolaffio, 1908.]

This product was isolated from egg yolk. The yolk was treated with acetone and the residue extracted with hot alcohol. On cooling neottin fell out and was purified by recrystallisation from alcohol.

On analysis it gave the following figures:—

C = 67·57 per cent.; H = 11·52 per cent.; N = 2·81 per cent.; P = 2·07 per cent., equivalent to the empirical formula $C_{84}H_{172}N_3PO_{15}$.

Neottin is simply another name for the so-called "carnaubon" of Dunham and Jacobson [1910]. A reference to the composition of the different specimens of carnaubon isolated (p. 145) shows that Fränkel and Bolaffio's analytical figures agree well with those obtained for carnaubon. Carnaubon, as shown by MacLean [1912, 1] and by Rosenheim and MacLean [1915], was a mixture of sphingomyelin and cerebrosides which sometimes contained some nitrogenous impurities. It is interesting to note that Dunham and Jacobson at first considered that their product was a triamino-monophosphatide. There is no further use for the term "neottin".

Group 4.—Triamino-diphosphatides. N : P = 3 : 2.

Under this group there are two substances both of which were isolated by Fränkel: one of these was prepared from kidney and the other from brain. To the brain product Fränkel gave the name sahidin. That both these products were mixtures is certain.

1. Kidney Triamino-diphosphatide.

[Fränkel and Nogueira, 1909.]

This substance was prepared by extracting kidneys, dried at 100°, for four hours with acetone. The residue was further extracted with alcohol, and the united precipitates obtained from both acetone and alcohol extracts dissolved in ether. From this ethereal solution, kephalin was separated by treatment with excess of absolute alcohol, and the kephalin-free filtrate precipitated with alcoholic cadmium chloride solution. The substance obtained in this way was dissolved in hot dry benzene and the triamino-diphosphatide precipitated from the benzene solution by the addition of alcohol.

The substance had a N : P ratio of 3 : 2 and gave on analysis :—

$C = 47{\cdot}77$ per cent. ; $H = 6{\cdot}87$ per cent. ; $N = 2{\cdot}03$ per cent. ; $P = 3{\cdot}04$ per cent. ; $O = 17{\cdot}35$ per cent. ; $Cd = 11{\cdot}55$ per cent. ; $Cl = 11{\cdot}11$ per cent. ; corresponding to the formula $C_{78}H_{133}N_3P_2O_{21}Cd_2Cl_6$.

From the method of isolation, it is quite obvious that this product of Fränkel was chiefly a mixture of lecithin and carnithin. The alcoholic solution, from which the substance was precipitated by cadmium chloride, must have contained both these substances, and since carnithin forms a compound with cadmium chloride which is soluble in hot benzene and is precipitated from this solution by alcohol, the composition of Fränkel's mixture admits of no doubt. No triamino-diphosphatide has as yet been separated from kidneys or from any other organ.

2. Sahidin.

[Fränkel and Linnert, 1910.]

Sahidin was obtained in the form of its cadmium chloride combination by fractionating petroleum ether extracts of brain with alcohol. After treatment with lead acetate, the sahidin was precipitated with cadmium chloride, and the cadmium chloride combination treated with hot benzene and alcohol as in the kidney product just described.

On analysis this compound gave:—

C = 48·81 per cent.; H = 8·31 per cent.; N = 2·14 per cent.; P = 3·15 per cent.; Cd = 16·59 per cent.; Cl = 10·47 per cent.; corresponding with the formula $C_{80}H_{167}N_3P_2O_{12} . 3CdCl_2$.

From a consideration of its method of preparation there is no reason to believe that such a compound as sahidin exists. Like Fränkel's other new compounds, it was a mixture in which lecithin and carnithin must have played a prominent part. There is no further reason for retaining the term "sahidin".

Group 5.—Decamino-diphosphatides. N : P = 10 : 2.

Leukopoliin.

[Fränkel and Elias, 1910.]

This substance was obtained as the cadmium chloride compound by precipitating the acetone extract of brain with cadmium chloride solution.

It had the empirical formula $C_{374}H_{720}N_{10}P_2O_{74} . CdCl_4$ and represented a decamino-diphosphatide or perhaps a pentamino-monophosphatide. Leukopoliin appears to have been chiefly composed of carnithin with some lecithin and probably other substances. There is no evidence whatever that this body has any claim to be considered as a chemical unit.

The above are the chief products the existence of which is either doubtful, or already disproved. One other substance—jecorin—still requires consideration.

Jecorin.

Jecorin was the name given by Drechsel [1886] to a product which he isolated from horse liver. The liver was minced, extracted with absolute alcohol, the alcoholic extract concentrated at 40° to the consistency of a syrup and the syrup treated with absolute alcohol until no more substance dissolved. The residue was then taken up in ether and from the ethereal solution jecorin was precipitated by the addition of absolute alcohol. On further purifying this jecorin, a yellowish-brown mass was obtained, which, on drying, formed a very hygroscopic yellowish-white powder. This powder was insoluble in absolute alcohol and in dry ether, but dissolved in wet ether. In water it first swelled up and then dissolved. When tested with

Fehling's solution a well-marked reduction was obtained. On analysis, jecorin was found to contain carbon, hydrogen, nitrogen, sulphur, phosphorus and sodium. A similar product was isolated from the liver of the dolphin [Drechsel, 1896].

Drechsel's observations were confirmed by Baldi [1887] who using an almost identical method prepared jecorin from rabbit and dog livers and from other organs.

Jecorin was also found in blood, but its alleged presence in this tissue was based more on conjecture than on actual experiment. In investigations carried out on blood prior to Drechsel's discovery of jecorin, it was found that blood contained, besides fermentable sugar, other reducing non-fermentable substances [Otto, 1885]. The nature of these substances was unknown, but on account of certain supposed superficial resemblances to jecorin it was assumed that they were identical with jecorin [Jacobsen, 1892, 1895; Henriques, 1897; Bing, 1898, 1899; Mayer, 1906, 1; Letsche, 1907]. No analytical figures were furnished by any investigator, and the alleged existence of jecorin in blood was based on entirely valueless observations. Indeed, it is known that some, at least, of the so-called jecorins of blood consisted of glycuronic acid compounds [Mayer, 1906, 1].

Jecorin, or a similar body, has been found in spleen and in brain [Baldi, 1887], in muscles [Baldi, 1887; Erlandsen, 1907], in heart [Erlandsen, 1907], in adrenals [Manasse, 1895], in bone marrow [Otolski, 1907], and in bile [Hammarsten, 1902].

The chemical nature of jecorin was investigated by Manasse [1895] who, on hydrolysis of a specimen prepared from liver according to Drechsel's directions, obtained choline, fatty acids, glycerine, phosphoric acid, glycerophosphoric acid and glucose. Since these products, with the exception of glucose, were identical with those obtained from lecithin, Manasse concluded that jecorin was a lecithin complex containing glucose.

Manasse's statements were further confirmed by Bing [1898, 1899] who prepared an artificial lecithin-glucose complex by dissolving sugar in an alcoholic solution of lecithin. On evaporating the alcohol and extracting the residue with ether, it was found that the ether extract contained a product which gave a precipitate on the addition of alcohol. Since this product reduced Fehling's solution, Bing concluded that jecorin was lecithin-glucose which contained small amounts of other substances as impurities. Unlike jecorin, Bing's artificial lecithin-glucose was apparently more soluble in alcohol than this body, and could be precipitated from alcoholic solution by excess of ether.

Mayer [1906, 1], in extension of Bing's work, isolated the artificial lecithin-glucose body, by precipitation of its ethereal solution with alcohol. He obtained products containing various amounts of sugar up to 84·5 per cent., and concluded that jecorin was a mixture of lecithin-glucose complexes containing varying amounts of sugar. A specimen of liver-jecorin containing about 14 per cent. of sugar was shown by Baskoff [1908] to contain the same amount of sugar after repeated precipitation by alcohol from its ethereal solution: similarly Meinertz [1905, 1] showed that a sample of liver-jecorin which also contained about 14 per cent. of sugar, had the same sugar percentage as the original product, after repeated precipitation with alcohol.

Though the investigations of Bing and others strongly suggested that jecorin was not a unit substance, the impossibility of changing the sugar content of jecorin by repeated precipitation by alcohol, as shown by Meinertz and by Baskoff, seemed to furnish some ground for regarding the product as something more than a mechanical mixture. Certain observers, indeed, still firmly held the view that jecorin was a definite chemical compound [Waldvogel, 1903; Waldvogel and Tintemann, 1904, 1906; Letsche, 1907], while others such as Meinertz [1905], Siegfried and Mark [1905], Mark [1907] as firmly believed that it was a mixture. Later observers [Leathes, 1910, Bang, 1911] concluded that there was no reliable evidence on which to form a definite opinion as to whether jecorin was a mixture or a chemical entity.

Jecorin a Mixture.

In considering whether jecorin represents a unit substance or a mixture it is important to compare the analytical data published by different observers. The figures show that the different products which the various investigators called jecorin were by no means identical substances, even when derived from the same organ.

Observer.	Source of Jecorin.	C.	H.	N.	S.	P.	Na.	Ratio P : N.
Drechsel [1886]	Horse liver	51·5	8·2	2·9	1·5	3·5	2·7	1 : 1·8
Baldi [1887]	Dog ,,	46·8	7·9	4·5	2·4	2·5	3·7	1 : 3·8
Siegfried and Mark [1905]	Horse ,,	39·7	6·4	5·2	2·2	1·9	5·9	1 : 6·0
Mayer [1906]	,, ,,	55·8	4·4	2·6	1·5	1·4	3·5	1 : 4·1
Baskoff [1908]	,, ,,	50·39	7·29	3·12	2·89	1·82	2·8	1 : 2·3

The variability of these results is so great that jecorin at once loses all claim to be considered as a unit body. They show the kind of figures that might be expected from a variable mixture in which the exact amount of the different compounds present depended on certain variable factors operating in the course of its preparation.

Further evidence of the non-unit nature of jecorin is afforded by statements as to carbohydrate contents of various preparations.

Mayer's [1906, 1] jecorin contained 18·2 per cent. of sugar, while that of Meinertz [1905, 1] and of Baskoff [1908] had about 14 per cent. On the other hand Offer [1904] obtained from liver a specimen which was carbohydrate free. Some jecorins did not reduce Fehling's solution directly [Offer, 1904; Meinertz, 1905; Waldvogel and Tintemann, 1906; Baskoff, 1908], while others reduced immediately [Drechsel, 1896; Baldi, 1887]. Adrenal-jecorin reduced only after being subjected to prolonged hydrolysis with sulphuric acid [Manasse, 1895]. Sakaki [1913; 1913, 1] obtained similar results with placenta-jecorin which he thought was of the same nature as Manasse's substance. How widely the two substances differed, is obvious from the fact that the N : P ratio of Manasse's product was 3 : 2, while that of Sakaki's was about 1 : 6. Indeed, from a survey of the literature, it seems that no two samples of jecorin ever furnished similar analytical data, and even the statements as to solubility vary considerably [Siegfried and Mark, 1905].

It is also significant that Baskoff [1908] could not obtain jecorin from the liver when the organ was treated according to Erlandsen's method [1907]. From this it would appear that jecorin is an artificial product formed during the processes described by Drechsel for the isolation of this substance. This view is in accord with the results of Waldvogel [1903] and Waldvogel and Tintemann [1904], who found much more jecorin in autolysed livers and in livers of dogs poisoned by phosphorus, than in normal livers [see also Meinertz, 1905]. According to Baskoff [1909, 1] alcohol poisoning does not affect the jecorin content of the liver, but markedly reduces the lecithin content.

Further, it was shown by Siegfried and Mark [1905] that jecorin could be divided into various fractions of very different composition. Two fractions obtained from jecorin by means which could not cause any decomposition of a chemical product, gave the following figures:—

Fraction I.	*Fraction II.*
C = 27·8	C = 39·7
H = 4·4	H = 6·4
N = 1·8	N = 5·2

The first fraction was obtained by dissolving jecorin in water and separating the precipitate which formed on the addition of methyl alcohol: the second fraction was separated from the aqueous methyl alcohol filtrate by the addition of ethyl alcohol. These observers also divided jecorin into five fractions by somewhat different methods, and obtained the following variations in the different fractions :—

N = 4·59 to 1·45.
P = 3·16 „ 0·38.
S = 1·84 „ 0·39.
Na = 5·10 „ 1·13.

These observations furnished strong evidence that jecorin was a mixture, and the only point which seemed somewhat difficult to reconcile with this view was the fact already mentioned that certain preparations of liver-jecorin contained a definite amount of sugar (about 14 per cent.), and that the sugar could not be removed, or its percentage reduced, by precipitating jecorin from its ether solution by means of alcohol. These difficulties were more apparent than real, for it is well known that certain phosphatides, such as lecithin, have the peculiar property of forming physical complexes with many organic substances [Bing, 1901]. It is also recognised that these complexes cannot be readily separated into their constituents by repeated precipitation from their solutions by means of alcohol. If, therefore, the concentration of sugar in a liver was such, that lecithin, or some other phosphatide present, took up about 14 per cent., one would expect no change in the sugar percentage after several precipitations with alcohol. That this particular amount of sugar was found in two or three different samples of liver-jecorin was obviously accidental, since some samples of jecorin from liver contained much more sugar, namely, 18·2 per cent. [Mayer, 1906, 1] and others no sugar at all [Offer, 1904]. Neither the amount nor the behaviour of sugar of jecorin furnishes any evidence in favour of the unit nature of jecorin, while all the other evidence recorded is only to be reconciled with the view that jecorin is a mixture. According to Meinertz [1905, 1] the sugar of jecorin can be removed by dialysis, and Scott [1916] has made the interesting observation that the sugar of lecithin-glucose can be obtained free in solution, by removing the lipin with colloidal iron.

The Nature of Jecorin.

From the method of preparation of jecorin, as given by Drechsel, it is quite certain that this substance must have contained large amounts of kephalin. Though kephalin is comparatively insoluble in alcohol, it appears to be relatively soluble in alcoholic solutions containing lecithin, and is always present in considerable amount in alcoholic extracts of tissue. Indeed, Drechsel's description of the physical properties of jecorin would do very well for kephalin. Like kephalin it was insoluble in absolute alcohol: it was also insoluble in dry ether, but dissolved in *wet* ether, properties which Parnas [1909] showed to be characteristic of kephalin. Like kephalin, jecorin first swelled up in water and then "dissolved". Jecorin contained sodium and so does kephalin [Thudichum, 1884; Parnas, 1909].

Although jecorin was supposed by Bing and others to be lecithin-glucose, it is clear that very little lecithin could be present, since this lipin must have been removed by the alcohol treatment which the jecorin invariably underwent.

It is quite certain that the bulk of jecorin must have consisted of kephalin, and the presence of sugar and other bodies is easily explained by the fact that kephalin, like lecithin, has a very marked tendency to form physical complexes with various organic substances [Fränkel and Neubauer, 1909; Thudichum, 1884].

Thus in the presence of sugar the formation of kephalin-glucose was to be expected. Jecorin represents not lecithin-glucose but kephalin-glucose [Frank, 1913], though no doubt small amounts of lecithin-glucose might be present. Another substance that must have been present to some extent is carnithin.

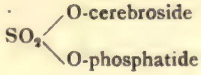
Jecorin is therefore to be regarded as a variable mixture consisting chiefly of kephalin and inorganic salts, together with certain organic substances (cerebrosides, "protagon" sugar, glycogen, etc.), which owing to adsorption, intersolubility and other physical phenomena, cannot be readily separated from the kephalin.

Indeed, from what we now know of the behaviour of the phosphatides, jecorin is exactly the kind of body we should expect to obtain as an artificial product, on treating liver according to Drechsel's directions for the preparation of jecorin.

The Sulphatides of the Brain.

Thudichum [1884] first postulated the existence of a lipin in which the phosphoric acid was replaced by sulphuric acid. He obtained from human brain samples in which the ratio sulphur : phosphorus was 3 : 2 and 1 : 1 respectively, but he was unable to further separate these substances.

Koch [1907, 1] claimed to have separated a compound containing the sulphuric acid joined to the sugar in an ester-like combination, represented by the formula,



$$SO_2\begin{cases}\text{O-cerebroside}\\ \text{O-phosphatide}\end{cases}$$

A sulphur-containing lipin entirely free from phosphorus, was isolated by Levene [1912] from ox brain. It resembled the lipins in its physical properties, was dextro-rotatory and melted at 210°. Levene is carrying out further work on this substance. At present it is not sufficiently characterised to be regarded as a chemical entity.

The elementary analyses obtained by Thudichum, Koch and Levene respectively are shown in the following table :—

	Thudichum.	Koch.	Levene.
C.	47·70	—	60·90
H.	7·54	—	10·67
N.	1·14	—	2·31
P.	3·94	1·80	0·00
S.	6·19	1·91	2·66

CHAPTER VII.

PLANT LIPINS.

THE difficulties encountered in isolating pure preparations of lipins from vegetable tissues, appear to be much greater than is the case with animal organs. The chief obstacles encountered in the investigation of plant products of this nature, are, to a great extent, dependent on the presence in the plant of large amounts of carbohydrate material which tend to form physical complexes of a "jecorin"-like nature with the lipins. It would also seem that certain of the methods employed in the case of animal tissues are unsuitable for the preparation of plant lipins. Our present knowledge of plant lipins is extremely limited, and we do not yet know with any certainty whether or not plant and animal lipins are identical. So far as it is possible to judge from the available literature, there is a certain amount of evidence pointing to the conclusion that no essential difference exists between the lipins of the two kingdoms. This view is by no means universally accepted, for certain observers assert that plant lipins are entirely different from those of animal origin. Thus Bang [1911] makes the following observations on this point. "Die Pflanzenphosphatide unterscheiden sich sehr deutlich von der tierischen. Lecithin kommt, aller Wahrscheinlichkeit nach, im Pflanzenreiche nicht vor, und ebensowenig finden sich andere aus dem Tierkörper bekannte Phosphatide."

The question, as to the nature of plant lipins, opens up what promises to be a fertile field for future research, for, up to the present, the investigation of these bodies has been carried out by a comparatively small number of workers.

The application to plants of some of the newer methods used in the case of animal organs, might well result in a solution of some of the above problems connected with this subject. The principal observations on plant lipins hitherto carried out, have dealt almost entirely with plant phosphatides.

PLANT PHOSPHATIDES.

The earliest indications of the presence of bodies of a phosphatide nature in plants were afforded by the observations of Knop [1860] and of Töpler [1861] who isolated from certain plant tissues fatty extracts containing phosphorus. Shortly afterwards Hoppe Seyler [1867] isolated a small amount of a substance closely resembling egg lecithin from the seeds of *Bertholletia excelsa;* a similar product was obtained from peas, but in neither case was the lipin prepared in pure form.

From this time onward until quite recently it was taken for granted that the only phosphatide present in plants was "lecithin". That a phosphorus-containing lipin was of general occurrence in plant seeds was shown by Heckel and Schlagdenhauffen [1886], but the first definite indication of the similarity of this body to lecithin was afforded by the work of Jacobson [1889], who showed that one of the decomposition products furnished by plant phosphatides was choline. Shortly afterwards Schulze and Likiernik [1891] obtained from seeds, lecithin-like substances which gave, on hydrolysis, choline, glycerophosphoric acid and fatty acids.

Estimations of Plant Lecithin.

In earlier investigations on plant lecithin much of the work consisted in estimating the amount of this product present in seeds and in other parts of plants. These estimations were carried out by extracting the tissue with ether and estimating the phosphorus percentage in the ether extract; the lecithin content was then calculated from the amount of phosphorus found. By this method Töpler [1861] obtained the following data from air-dried seeds:—

	Percentage of Lecithin in Ether Extract.	Percentage of Lecithin in Dried Seeds.
Beans . . .	18·75	0·30
Vetches . . .	13·02	0·39
Peas . . .	30·46	0·61
Lupines . . .	7·55	0·37

Some results obtained by Beyer [1871] and later by Jacobson [1889] suggested that this method by ether extraction was unsuitable for quantitative purposes.

Jacobson extracted seeds with alcohol and estimated the phosphorus in the ether-soluble part of the residue from the alcoholic extract. He found that this method yielded much higher results than the method of direct ether extraction, as may be seen from his figures :—

	Percentage of Lecithin in Fat Obtained. (Calculated from P. per Cent.)
Beans	64·04
Vetches . . .	20·83
Peas	50·25
Lupines . . .	50·00

While Jacobson's observations were quite correct, his explanation of them was wrong, for he concluded that the excess of phosphorus obtained by alcohol treatment was due to the extraction by alcohol of some ether-soluble phosphorus-containing body other than lecithin.

The true explanation was afforded by Schulze and Steiger [1889], who showed that treatment of finely powdered seeds with ether dissolved only part of the lecithin present: to obtain the whole of the lecithin, extraction with hot alcohol was necessary. That the alcoholic extract of seeds which had already been treated with ether, actually contained lecithin and not some other phosphorus compound, was inferred by these observers from the fact that this extract yielded fatty acids and choline. This behaviour of seeds towards ether was explained on the assumption that part of the lecithin existed in combination with some other substance in the seeds, and that this compound was decomposed by hot alcohol [see also Maxwell, 1891].

A number of quantitative estimations of various seeds were carried out by ether-alcohol extraction [Schulze and Steiger, 1889; Stellwag, 1890; Bitto, 1896; Stutzer, 1908; Vogeler, 1909, and others]. The results indicate that, on the whole, the amount of lecithin present in plants is small.

Seeds.	Percentage of Lecithin Calculated from P. of Ether-alcohol Extract [Schulze and Steiger].
Lupinus luteus . . .	(1) 1·55 (2) 1·59
Soja Hispida	1·64
Vicia Sativa	1·22
Faba vulgaris . . .	0·81
Triticum vulgare . . .	0·65
Secale cereale . . .	0·57
Hordeum distichon . .	0·74
Linum usitatissimum . .	0·88

Further observations on the amount of lecithin in plant seeds are given by Schulze and his collaborators, also by Stromer and Fallada [1906], by Deiler and Fraps [1910], and by Parrozzani [1910].

It is obvious that the accuracy of this method of lecithin estimation depended on certain hypotheses :—

1. That the extraction of the seeds was complete.

2. That the whole of the phosphorus found in the ether-alcohol extract of seeds was present in lipin combination, and that only one lipin (lecithin) was present.

The first of these hypotheses was combated by Bitto [1894], who maintained that the extraction of seeds by ether and alcohol according to Schulze and Steiger's method was incomplete. Schulze [1894] contended that Bitto's criticisms had but little weight, since seeds which had been treated by this method contained only small traces of phosphorus. Later [1904] he remarked that it was not quite certain whether alcohol at 60° completely extracted the lecithin from seeds, but to guard against decomposition he still recommended the use of alcohol at this temperature.

Schulze came to the conclusion that all the phosphorus of the alcohol and ether extracts was probably represented by lecithin, for no other phosphorus-containing substance, with the exception of phosphates, could be found in plants. Since phosphates were insoluble in both alcohol and ether, they could be excluded. He did not, however, entirely exclude the possibility that some phosphorus might be present, which was not represented by lecithin, and stated that in any case the method gave maximum results [Schulze and Steiger, 1889; Schulze and Frankfurt, 1894; Schulze, 1904].

It was only in recent years that the presence of lipins, other than lecithin, in plant extracts was recognised, and the more general term phosphatides used instead of lecithin [Schulze and Winterstein, 1903; Winterstein and Hiestand, 1906; Schulze, 1908]. While this method of estimation gave a general idea of the lipin content of the tissues examined, it was by no means an ideal one, and the figures obtained could only be regarded as a very rough index of the lipin content.

Schulze and Likiernik [1891] modified the above method for obtaining and estimating lecithin. After treatment of the finely ground seeds with ether and then with alcohol at 50 to 60°, they dissolved the residue obtained from the ether-alcohol extract in ether, washed the ether solution with water and finally evaporated the washed ether solution. The residue was "crude lecithin" from which a purer product was obtained by dissolving in hot spirit and cooling the solution, when an amorphous body separated which had the properties of lecithin [see also Schulze and Frankfurt, 1894; Stoklasa, 1895; Schulze, 1897, 1898; Schulze and Winterstein, 1910].

An improvement of this method was introduced by Schulze [1908], who suggested that the alcoholic solution of "raw lecithin" obtained in the manner described should be evaporated and the residue treated with acetone. What remained after acetone extraction was then dissolved in ether, and lecithin precipitated from this solution by methyl alcohol.

Though no reliable method for the estimation of plant phosphatides has as yet been evolved, the results recorded by different observers indicate that the leguminosæ are relatively rich in this substance, the dried seeds containing up to 1·5 per cent. or more of phosphatide. On the other hand, cereals generally contain much less, the amount present being roughly about 0·5 per cent.

Observations on the Nature of Plant Phosphatides.

Schulze and Winterstein [1903] drew attention to the marked variability in the phosphorus percentage of different specimens of plant lecithin as found by various observers, and pointed out, that while the theoretical formula for lecithin required a phosphorus content of about 3·8 per cent., many plant lecithins contained considerably less than this amount. Similar observations applied to the nitrogen content. This variation in phosphorus percentage is well seen in the figures given:—

Observer.	Source of Lecithin.	Phosphorus Percentage.
Schulze and Likiernik [1891] .	Lupines and vetches . .	3·68
Stoklasa [1895]	Oats (germinating) . .	4·23
Schulze and Frankfurt [1894] .	Mushroom (Boletus edulis) .	3·41
,, ,, ,, ,, .	Rye and barley . . .	*Circa* 2
Wintgen and Keller [1906] .	Soya beans (brown) . .	2·96
,, ,, ,, ,, . .	,, ,, (black) . .	2·51
Euler and Nordenson [1908] .	Carrots	2·53 to 2·75

With the exception of Stoklasa's specimen, the phosphorus data are all too low for lecithin, though, in the case of lupines and vetches, the percentage approaches the theoretical figures. The obvious inference from this was either that plant lecithin differed in constitution from that obtained from animals or that it contained some impurity. Considerable light was thrown on the problem by the important discovery of Winterstein and Hiestand [1906] that plant phosphatides yielded on hydrolysis not only the usual decomposition products of lecithin, but sugar as well. The sugar content was often very large and, in some cases, amounted to over 16 per cent.; in other cases, only a small amount was present, and in one product (lecithin from the seeds of *Pinus cembra*) sugar was probably absent [Schulze, 1907, 1908, 1].

It is interesting to note that the specimens of lecithin containing small amounts of phosphorus generally yielded the largest amount of carbohydrate, a result which indicated that the sugar accounted, at least to a large extent, for the low phosphorus value. Schulze [1907] showed that the presence of free sugar was not the sole cause.

Observer.	Source of Phosphatide.	Percentage of P.	Percentage of Sugar.
Winterstein and Hiestand [1908] .	Wheat meal . .	1·6 to 2·5	16·65
,, ,, ,, ,, .	Avena sativa seeds .	1·96	16·81
,, ,, ,, ,, .	Lupinus albus seeds	2·7	17·9
,, ,, ,, [1906, 1908]	,, luteus ,,	3·69	1·1
,, ,, ,, ,, ,,	Vicia sativa seeds .	3·67	3·16
,, ,, ,, ,, ,,	Pinus cembra seeds .	3·60	Traces
,, ,, ,, [1908] .	Potato tubers . .	1·61	6·98
,, ,, ,, ,, .	Pollen . . .	1·44	14·93

The sugar present seemed to consist of glucose, galactose, pentose and methyl pentose; some of it could be removed from the lecithin by repeated washing with water, but it was impossible to obtain sugar-free products by this means. That some, at any rate, of this sugar was present in firm chemical combination, and not as a physical complex, was indicated by the fact that it was necessary to boil the lecithin for several hours with 5 per cent. sulphuric acid in order to split off all the carbohydrate [Hiestand, 1906; Schulze, 1907; Winterstein and Hiestand, 1906, 1908].

The presence of carbohydrates in plant phosphatides appears to be almost universal, and suggests that we are here dealing with products of the nature of so-called "jecorin," but that, along with such physical complexes, bodies identical with, or similar to, the animal cerebrosides are of widespread occurrence. According to Winterstein and Smolenski [1909] these combinations exist preformed in the plant and are not artificial products of extraction. Various attempts at fractional separation of the different lipins found in plant extracts have been attempted, and the isolation of crystalline phosphatides claimed [Winterstein and Smolenski, 1909; Smolenski, 1909], but up to the present, there is no evidence that any observer has succeeded in obtaining from plants, a carbohydrate-free lecithin similar to the body found in animal tissues and organs.

That plant phosphatides tend to take up inorganic material appears from the observations of Schlagdenhauffen and Reeb [1902], and of Winterstein and Stegmann [1909].

Hydrolytic Products of Plant Phosphatides.

In general, the decomposition products obtained on hydrolysis of plant phosphatides are similar to those found in lecithin from the animal kingdom, and include fatty acids, glycerophosphoric acid and bases.

Fatty Acids of Plant Phosphatides.

As in the case of animal lecithin, little is known with certainty as to the nature of the fatty acids of plant lecithin. Schulze and Likiernik [1891] found oleic acid and a solid acid among the products of hydrolysis of a phosphatide prepared from leguminosæ seeds. The solid acid probably consisted of a mixture of palmitic and stearic acids. Similar acids were found by Shorey [1898] in sugar-cane lecithin. Njegovan [1911] also isolated from *Lupinus albus* phosphatide what appeared to be a mixture of palmitic and stearic acids: indications of the presence of unsaturated acids were also obtained. No careful and systematic investigation of plant phosphatide fatty acids has as yet been carried out, but the small amount of information we possess suggests that they are probably of the same nature as the fatty acids of animal lecithin.

Bases of Plant Phosphatides.

The general presence of choline in plant phosphatides has been established by the work of many observers, and there is little doubt that this body is a universal constituent of these substances. Since Trier's [1911] interesting observation that amino-ethyl alcohol[1] ("colamine") was present in the phosphatides of bean meal (*Phaseolus vulgaris*), evidence has been obtained that this base is of widespread occurrence in plant phosphatides [Trier, 1912; 1913, 2; 1913, 3].

Besides choline and "colamine" various other bases have been found from time to time in plant lipins, but so far, these are the only two products for which proof has been furnished that they actually form part of the lipin molecule. Though other bases are no doubt sometimes present, they probably occur, not as an integral part of the phosphatide molecule but as impurities which are difficult or impossible to separate [Engeland, 1909; Schulze and Pfenniger, 1911]. Among such substances are betaine and trigonelline. Betaine is often found in plant lipins [Schulze and Pfenniger, 1911; Schulze and Trier, 1911] and has been considered by several observers to be part of the phosphatide complex [Shorey, 1898; Lippmann, 1887]. Though generally present in comparatively small amount, it sometimes occurs in considerable quantity, and in a lecithin preparation isolated by Lippmann [1887] the only base found was betaine.

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[1] Trier [1912, 2] considers that choline is formed in the plant by the methylation of amino-ethyl alcohol.

A new base isolated by Njegovan [1911], and called by him "vidin," was according to Trier [1912] a mixture of choline with ammonium salts.

Glycerophosphoric Acid of Plant Phosphatides.

Glycerophosphoric acid has been identified by many observers in phosphatides isolated from various plants. Zinc glycerophosphate was prepared by Schulze and Likiernik [1891] from lecithin isolated from leguminous seeds. Hiestand [1906] prepared barium glycerophosphate from plant lecithin, but was unable to decolorise his product sufficiently for polarimetric observations. The difficulty in obtaining a pure product from plant lecithin is much greater than is the case, for instance, from egg lecithin: from the latter source barium glycerophosphate can be very easily isolated in the form of a snow-white powder, while from plant lecithin, the salt is obtained with difficulty and, even then, is generally more or less highly coloured and hygroscopic [Trier, 1913, 1].

The barium compound prepared from egg lecithin by Willstätter and Lüdecke [1904] contained 43·41 per cent. of barium, and was presumably represented by the formula $C_3H_7PO_6Ba + \frac{1}{2}H_2O$. From wheat meal phosphatide, Winterstein and Hiestand [1906] obtained a barium salt with 39·5 per cent. of barium to which these observers ascribed the formula $C_3H_7PO_6Ba + 2H_2O$; this substance differed therefore from that of Willstätter's in having 1½ molecules more water of crystallisation.

Njegovan's [1911] barium glycerophosphate was not very different in barium content from Willstätter's products, but, unlike these preparations, was optically inactive and dissolved in water with difficulty.

Trier [1912] succeeded in obtaining an optically active *dextro-rotatory*[1] barium glycerophosphate from plant lecithin, which in its barium content and solubility in water closely agreed with the dextro-rotatory glycerophosphate isolated from brain by Fränkel and Dimitz [1910].

On the whole, it would seem that the glycerophosphoric acid of plant phosphatides does not differ from that of animal phosphatides.

Relation of Plant and Animal Phosphatides.

The above observations support the view that the phosphatides of plant and animal organisms are similar substances. Obviously our present limited knowledge does not permit a definite statement on this point, but the apparent differences between animal and plant lipins, while probably largely due to incomplete investigation, depend also to some extent on incorrect interpretation of experimental results [Trier, 1913].

Relation of Phosphatide Content to Protein Content of Seeds.

According to Schulze and Likiernik [1891], Schulze, Steiger and Maxwell [1891] the amount of phosphatide present in seeds varies directly with the protein content. In seeds with a low protein content less phosphatide occurs, while on the other hand, an increase of protein is accompanied by an increase of phosphatide. This observation was confirmed by Stoklasa and seems to be borne out in a general way by his figures, some examples of which are quoted (Czapek, 1913):—

	Lecithin Percentage.	Protein Percentage.
Triticum vulgare . . .	0·65	12·04
Zea Mais	0·28	9·12
Pisum sativum . . .	1·23	23·15
Vicia Faba	0·81	25·31
Glycine Hispida . . .	1·64	32·18
Papaver somniferum . .	0·25	19·53
Helianthus annuus . . .	0·44	14·22

Trier [1912] sought to explain this relationship of phosphatide to protein by assuming that the amino acids of protein and the alcohol radicles of phosphatides were formed simultaneously from aldehydes according to the Cannizzaro reaction. It is by no means certain that this relationship really exists, for the phosphatide estimations on which its conception depends, were carried out by estimating the phosphorus in the ether-alcohol extract, and calculating the phosphatide from this value in the usual way. This method, as already stated, gives only rough results, but on the whole the figures obtained are suggestive.

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[1] Willstätter's glycerophosphate was lævo-rotatory.

Changes in Plant Phosphatide Content during Germination and Growth.

According to Schulze and Frankfurt [1894], unripe seeds contain less phosphatide than the ripe product. This observation is not in agreement with the results of McClenahan [1913], who found the very opposite to be the case. It is probable that the variations in the phosphatide content of seeds at successive stages of ripening differ with the particular seeds investigated.

With regard to the variations of phosphatide content in germinating seeds, many experiments have been carried out. Unfortunately, the results are not always in agreement, but, on the whole, it would appear that the amount of phosphatide present is greatly influenced by the conditions under which germination takes place. When seeds are allowed to germinate in darkness, the phosphatide apparently decreases [Schulze and Steiger, 1889, 1], while in the presence of sunlight it increases [Maxwell, 1891, 1].

Observer.	Seeds Grown in Darkness.			
	Seed.	Percentage of Phosphate in Seed.	Percentage of Phosphate in Seedlings.	Time During which Germination Proceeded.
Schulze and Steiger [1889, 1]	Lupinus luteus	2·10	0·44	14 days
Schulze [1893] . . .	Vicia sativa	0·74	0·19	4 weeks
Schulze and Frankfurt [1894]	Vetch seeds	0·74 to 1·22	0·86	—
Prianischnikow [1895] . .	Vicia sativa	1·08	0·58	10 days
Merlis [1897] . . .	Lupinus angustifolius	2·20	1·14	15 ,,

Similar results to the above were found by Zaleski [1902], Iwanoff [1902] and by Bernardini and Chiarulli [1910]. In the seeds of *Helianthus annuus* Frankfurt [1894] found 0·44 per cent. of phosphatide, while the etiolated plants contained 0·71 per cent.—a result contrary to the general experience of other observers, and similar to that obtained when seeds develop in the presence of light.

Observer.	Seeds Grown in Light.			
	Seed.	Percentage of Phosphate in Seed.	Percentage of Phosphate in Seedlings.	Time During which Germination Proceeded.
Maxwell [1891, 1]	Phaseolus vulgaris	0·933	1·841	Until radicle had protruded through testa about ¾ in.
Stoklasa [1895] .	Rape seed	0·45	5·22	5 days
,, ,, .	Beet seeds	0·45	1·78	9 ,,
,, ,, .	Buck wheat	0·51	1·03	8 ,,
Wallerstein [1897]	Barley	3·06 (of total fat content)	5·04 (of total fat content)	9 ,,

Green and Jackson [1905], and Bernardini and Chiarulli [1910], obtained data supporting the above results.

It must be remembered that all these figures are based on a method of determining phosphatide which is open to criticism, and that probably on this account, they are to be looked upon as giving general indications and not definite data of the actual changes in phosphatide content, which germinating seeds undergo.

PLANT CEREBROSIDES.

That bodies similar to the cerebrosides of animal tissues are of widespread occurrence in plants, is indicated indirectly by various experimental results. Thus the constant presence of sugar which in many cases appears to be galactose, and the difficulty with which this galactose is split off on hydrolysis, suggest the presence of cerebrosides.

Very few attempts have been made to isolate these bodies, but in one or two cases, products of the nature of cerebrosides have been obtained. Trier [1913, 3] isolated a substance having the properties of a cerebroside from rice, and similar bodies have been obtained from mushrooms [Bamberger and Landsiedl, 1905; Zellner, 1911]. The isolation of cerebrosides from plants should furnish a fertile field for future investigation.

CHAPTER VIII.

THE FUNCTION OF LIPINS.

Lipins from the Biological Standpoint.

FROM what has already been said regarding the unsatisfactory state of our knowledge of the lipins, it follows that their exact function in the animal and vegetable economy is necessarily obscure. Their great importance in the living organism is indirectly proved by their general occurrence in every cell, but little or no direct experimental proof indicating their specific function has as yet been obtained. When we consider the obscurity in which the chemistry of the lipins has been shrouded and the fact that even now their chemical nature, in many cases, is not satisfactorily established, it is easy to understand that many of the properties and functions ascribed to these bodies, are based on little more than imagination. A few years ago, when the subject of immunity was still in its earliest infancy, it was the fashion to ascribe to the action of "lipoids" many of the inexplicable phenomena encountered. Each author's interpretation of the term "lipoid" varied, and was often as difficult to understand as the phenomena he attempted to explain, until gradually the term became one of the most useful covering words in the new science. This extensive use of the word "lipoids" endowed these bodies with an artificial and one-sided importance which subsequent research has failed to substantiate, and it is now certain that the "lipoids" do not play the important rôle in the processes of immunity with which they were formerly credited. It is generally assumed that lecithin can act as an antigen in the Wassermann reaction, but from some unpublished experiments carried out by Dudgeon and MacLean it appears that this is not so. The active substance is not lecithin itself, but some other body which is difficult to separate from lecithin. This unknown substance can be separated under certain conditions, and very active solutions obtained which are practically lecithin-free. In fact, so far as can be ascertained from the more recent literature, it is questionable whether the lipins are concerned to any extent in immunity reactions. This does not, however, indicate that these complex substances are not as important as they were formerly supposed to be; it only shows that their particular functions are exercised in other directions. Were these bodies not essential for life, it is almost impossible to understand their invariable presence wherever the phenomena of life are manifest. That they are indispensable is obvious, but the particular part they play is at present quite unknown, despite the voluminous literature on the subject. A few of the alleged chief functions of the lipins are discussed below. In practically every case it will be seen that the evidence adduced is capable of explanation in other ways than the one usually adopted, and on the whole it is not too much to say that the biological significance of the lipins is so far a closed book. There is hardly a statement made on this problem by one investigator, which is not contradicted by some other observer, and the whole subject, in so far as experimental difficulties permit, requires thorough re-investigation. From the physical point of view the colloidal nature of the lipins is of importance in regulating certain processes in the cell; the presence of unsaturated fatty acids in the molecule is also suggestive, since the chemical reactivity of the molecule is thereby increased.

The Part Played by the Lipins in the Phenomena of Metabolism and Growth.

The addition to our knowledge of the composition of proteins which has taken place during the past twenty years, has shown us that the proteins are relatively stable compounds, and it has become customary to look elsewhere for substances of a high degree of lability which might play some part in conditioning the peculiar nature of living protoplasm. Bang [1911] for instance considers that the lipins are the most labile of all the components of the colloidal systems which manifest the properties of living matter, and that they play an important rôle in the phenomena of metabolism.

The question of the specific nature of the fatty acids of lecithin was for a long time assumed [Henriques and Hansen, 1903], but positive evidence has now been obtained [McCollum, Halpin and Drescher, 1912] that the degree of unsaturation of the lipin fatty acids of egg yolk is more or less accidental and can be influenced by variations in the diet. Two series of experiments were carried out on hens: in one of these, the hens were fed on a normal diet, and in the other, on a diet as far as possible freed from fat and lipins. The fats and lipins of the eggs laid by hens fed with the normal diet were markedly more unsaturated than those of the eggs from hens fed on the fat- and lipin-free food.

	Iodine number of :—					
	(*a*) *Fats.*			(*b*) *Lipins.*		
	(1)	(2)	(3)	(1)	(2)	(3)
Nearly lipin-free ration . .	50·0	54·36	51·1	35·22	34·07	34·0
Ordinary ration	63·2	65·5		63·7	63·1	

These experiments appear to establish definitely the important fact that the degree of saturation of the fatty acids of the lipins can be influenced by the character of the diet. It seems, indeed, highly probable that the lipins play an important, if as yet unknown, part in the history of fat metabolism [MacLean and Williams, 1909].

During the development of the chick from the egg, a very marked change in the distribution of the lipins takes place; as much as 65 per cent. of the total phosphorus is present in the egg in the lipin form: in the chick only 20 per cent. is so combined [Plimmer and Scott, 1909; Eaves, 1910]. From the large proportion of lipin present in egg yolk, it might be inferred that lecithin is a particularly suitable form of food for the developing animal.[1] Danilewsky [1895], for instance, found that frog's eggs placed in water containing ·07 per cent. of lecithin gained 300 per cent. more than those placed in ordinary water. Frog embryos when fed with egg lecithin have been found to increase in weight and size more rapidly than normal tadpoles [King, 1907; Johnson, 1913]. On the other hand, in the division rate of paramœcia [Browder, 1915] and in the development of fertilised sea urchin eggs in sea water [Robertson, 1913; Goldfarb, 1910] marked retardation of growth has followed the addition of lecithin to the culture medium. The growth of Flexner-Jobling carcinoma in rats is also markedly retarded by local injections of an aqueous emulsion of lecithin [Robertson and Burnett, 1913].

Many observers record the beneficial effects of lecithin administered as food. It is said to produce an increase in body weight and generally to act as a stimulating agent to normal growth [Desgrez and Zaky, 1902; Hatai, 1904; Bain, 1912]. Its administration is followed by a marked increase in the lecithin content of the muscles, and more especially of the liver where a high lecithin value may be found as long as fifteen days after the lecithin feeding has ceased [Franchini, 1907]. The retention of both phosphorus and nitrogen after lecithin feeding seems also to be fairly well established [Stassano and Billon, 1902; Slowtzoff, 1906; Yoshimoto, 1910; Patta, 1912]. Slowtzoff showed that this retention of nitrogen and phosphorus was accompanied by a diminution of the total amount of sulphate excreted, and hence concluded that the ultimate effect is the retention of protein in the body. Addition of lecithin to the diet is without effect on the secretion of milk [Fingerling, 1912, 1]. It is questionable whether any lecithin is absorbed as such when administered by the mouth; it is readily acted upon by lipase and is probably therefore broken down before it is absorbed. Desgrez and Zaky [1902, 1] describe an increase in body weight, especially in the nervous and skeletal systems, following the injection of lecithin, a result not confirmed by feeding experiments [Hatai, 1904]. On the whole, the results of feeding experiments with lecithin are inconclusive, and its administration does not appear to be followed by any very significant result.

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[1] According to Glikin [1907, 1] a high percentage of lecithin is found in the bone-marrow of the young of those species of animals, in which there is a helpless and ill-developed condition at the time of birth.

The most noticeable effect produced by the injection of lecithin into the blood appears to be a very marked increase in the number of red blood corpuscles and of the mono-nuclear leucocytes [Stassano and Billon, 1902; Kepinow, 1911; Bain, 1912].

For a long time it was believed that the lipins could not be synthesised by the organism, unless the phosphorus were supplied in an organic form. This view was disproved by the work of Gregersen [1911] who showed that the organism could maintain itself in phosphorus equilibrium when phosphates were the only phosphorus-containing material given in the food. Some interesting points about phosphorus metabolism are brought out by Gregersen; he found that the addition of phosphates to the food does not influence the loss of phosphorus from the organism when a nitrogen-free diet is given. If the diet contain nitrogen but be free from phosphorus, loss of phosphorus occurs; in the presence of calcium and magnesium salts the greater proportion of the loss occurs in the fæces; in the absence of these salts the phosphorus appears chiefly in the urine. Fingerling [1912, 1912, 1] found that ducks fed on a diet containing only inorganic phosphorus produced eggs with a normal lecithin value. The synthesis of lecithin from food containing only inorganic phosphates was therefore established, a result since confirmed by the work of Plimmer [1913] and McCollum, Halpin and Drescher [1912].

Experiments carried out on the effect of feeding animals with a fat-free diet have led to somewhat variable results. The constituents of the diet are first treated by extraction with solvents such as alcohol and ether, by which fats and lipins are removed. It is obvious that the results obtained may vary considerably according to the exact method of extraction employed and the completeness with which the fatty substances are removed; at the same time constituents other than fats and lipins may be extracted by the solvents chosen. Stepp [1909, 1911, 1913] found that previous extraction with solvents such as alcohol and ether removed something from the diet which was essential for the maintenance of life. He suggested that the essential substance removed was of a lipin nature, but he could not find any known lipin which restored the normal power of the diet. Osborne and Mendel [1912] found in experiments on rats that the body weight was well maintained on a fat-free diet. McArthur and Luckett [1915] were able to eliminate lecithin, kephalin, cerebrosides, cholesterol and fat from the indispensable constituents of a food for mice. They found, however, that a substance was present in egg yolk, soluble in cold alcohol but insoluble in ether which when added to their "synthetic fat-free" food made a complete diet.

The interesting work undertaken during the past ten years on the etiology of such diseases as beri-beri and scurvy has shown the influence of certain unknown substances, present only in minute quantities, but essential to the maintenance of normal nutrition. It was at one time claimed that lecithin produced a curative effect in beri-beri [Schaumann, 1911], but the curative effect has been found to disappear as the lipins became more highly purified [MacLean, 1912; Funk, 1911, 1912; Cooper, 1914].

The remarkable work recently carried out on the diet necessary to produce growth in young animals [Hopkins, 1912] showed the importance of specific substances which were absolutely essential for the production of growth in young animals, but without which, normal life could be maintained for a long time in adult animals. Milk was found to be especially active in restoring the power of growth to young animals fed on a synthetic diet. Butter was also found to possess the same property [McCollum and Davis, 1913; Osborne and Mendel, 1913, 1; 1916], and the essential constituent was found to be still contained in purified butter fat which could be shown to be entirely free from lipins. It appears, therefore, that the substance essential to growth is not of a lipin nature; it is contained in milk and being soluble in the butter fats is separated with them.[1] There is, indeed, some evidence to suggest that the nature of the influence of lecithin on growth is rather of an inhibitory character. Large doses of lecithin administered to mice are stated to have produced a retardation of growth; it is, however, so slight that it is questionable whether it can be regarded as significant [Robertson, 1916]. A slight retardation of the growth of suckling mice also followed the administration of 100 mg. of egg lecithin per day given by mouth to the mother [Robertson and Cutler, 1916].

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[1] Robertson (J. Biol. Chem., **24**, 1916, 397, 409) has prepared a substance, which he calls Tethelin, from the anterior lobe of the pituitary gland by precipitating its alcoholic

The determination of the amount of lipins present in the body glands has led to the interesting result that certain of the ductless glands contain a high proportion of lipins compared with tissues such as muscle-fibre. This is especially marked in the suprarenals and pituitary body and to a somewhat less extent in the pineal gland, the infant thymus, and the corpus luteum of pregnancy in its early stage. The proportion of lipins in the thyroid is, however, low [Fenger, 1916]. Young tissues seem to be particularly rich in lipins; this is noticeable in the spinal cord of young animals, the amount of lipin decreasing with age [Glikin, 1907]. The bodies of young animals contain a much higher percentage of alcohol-soluble phosphorus than those of the old, and the high lipin value of the corpus luteum in the early stages of pregnancy may either be an accompaniment of the active growing tissue or may be related with the specific secretory activity of the cells [Corner, 1917].

The Influence of the Lipins in Narcosis and in Certain Oxidative Processes.

Overton [1901] postulated that every living cell is limited by a semi-permeable membrane which consists essentially of lipins and which is disintegrated at the death of the cell. This membrane is impermeable to salts and regulates the passage of substances to and from the cell; in its absence, the exchange of substances between the cell and its surrounding medium would be conditioned only by the osmotic pressure of the substance. Overton suggested that a consideration of the distribution of an organic substance between oil and water would throw light on the ease with which the substance would pass into the cell. He determined the narcotic effect on tadpoles of a

extract with ether; it has a N : P ratio of 4 : 1 and gives on hydrolysis fatty acids and inosite. It has a marked effect on the growth of white mice, retarding growth between the 6th and 20th weeks and accelerating it from the 20th to 60th weeks. The evidence is at present insufficient to justify us in regarding Tethelin as a pure lipin, and the nature of the growth stimulating substance of the pituitary requires further elucidation.

large number of organic substances, and ascertained the smallest concentration of the narcotic which would produce narcosis. There was a striking parallelism between the numbers obtained in these experiments and the partial coefficients of distribution of the same substance between oil and water. He therefore argued that the power to produce narcosis depended entirely on the power of the substance to dissolve in the lipin membrane. Bang [1907] pointed out certain objections, as for instance that the reaction period between the paralysis of the spinal cord and of the medulla oblongata varied widely with different narcotics, and suggested that the narcotic must react chemically with the lipin membrane. The strength of a narcotic on Overton's theory would be proportional to the readiness with which it entered the lipin membrane, but a subsequent chemical action between the narcotic and the lipin or other constituent might of course take place. Though Overton's theory may probably contain a germ of truth, it has been found to be inconsistent with many ascertained facts, and can be no longer accepted in its original form.

Oxydases.

An interesting series of experiments has been carried out by Vernon [1912, 1913, 1914] which aim at showing the dependence of the action of oxidases on the cell-lipins and in thus differentiating them from nearly all other ferments. He investigated the production of indophenol-blue by the action of the oxidase present in the kidney on a solution containing *α*-naphthol and *p*-phenylenediamine, and found that if the tissue be first treated with an organic solvent at certain concentrations, the oxidase action is rapidly destroyed. The effects of a large number of organic solvents were investigated, and were found to be proportional to the molecular weight and independent of the homologous series to which the solvent belonged. The sudden nature of the change is shown by the fact that a concentration of 4 per cent. of acetone produces no effect on the oxidase, but one of 7 per cent. totally destroys its action. The concentrations of alcohols and ketones necessary to destroy 50 per cent. of the oxidase activity in Vernon's experiments are relatively twenty and twelve times as great as those necessary to produce narcosis in tadpoles. Vernon concluded that the effect of the oxidase is dependent on the lipins, perhaps on the lipin membrane which holds together the tissue-oxygenase and peroxidase and make possible their mutual enzymatic activity.

Czapek [1910] studied the effect of organic solvents on the exosmosis of tannin from vegetable cells and ascribed their action to surface tension phenomena; Vernon [1913] gave to Czapek's results a similar interpretation to his own, and suggested that they are also conditioned by the presence of a lipin membrane.

Respiration of Plants.

Somewhat similar experiments have been carried out by Palladin and Stanewitsch [1910] on the respiration of plants. They treated wheat embryos with organic solvents and then soaked them for a short time in water, estimating the amounts of carbon dioxide evolved during definite intervals. The respiration energy was lowest after treatment with those solvents which extracted most lipin.

The hypothesis of the lipin-cell membrane and of its close relationship to the phenomena of narcosis and of the oxidative and respiratory processes is an attractive one, but it is not yet clear that the experimental results obtained permit only of this explanation.

The Action of Lipins on the Digestive Ferments.

This problem has been studied by a number of observers with quite contrary results; for instance, the same lipins are described respectively as activating, inhibiting and having no influence on the diastatic reaction. Commercial lecithin or crude ethereal extracts of organs have been used as the lipin material, and in no case does any purification appear to have been attempted. A short summary of the results of various observers is appended, but at present there is no conclusive evidence that the pure lipin has any influence either in activating or inhibiting the action of diastase or of the proteolytic ferments.

Proteolytic Ferments.

Küttner [1907] found that small doses of lecithin exerted an inhibitory influence on the action of pepsin, somewhat greater doses increased the ferment activity, whilst still larger amounts again produced an inhibitory effect. The action on trypsin was also inhibitory, a result confirmed by Neumann [1908]. The lecithin used in these experiments was Kahlbaum's commercial lecithin.

Diastatic Ferments.

Centanni [1910] examined the influence of the ether-soluble constituents extracted from blood and from egg yolk on the diastatic action of the liver and found that the ferment activity was increased, especially by egg-yolk extract. This result he ascribed to the effect of phosphatides. Lapidus [1910] found that commercial lecithin produced an inhibitory effect on the action of ptyalin and of pancreatic diastase, but that it increased the diastatic effect of serum, when this had previously been diminished by treatment with ether. Starkenstein [1911] criticised unfavourably the technique of earlier observers, and concluded that the presence of lecithin did not influence the diastatic reaction, a conclusion supported by Terroine [1911]. Minami [1912], using an ethereal extract of different organs, found an activating effect on the diastatic reaction; commercial lecithin was found to produce an inhibitory action.

On the whole, there does not appear to be any reliable evidence that the lipins exert any specific influence on diastatic action, and the contradictory results obtained may well be due to the lack of precautions taken in the preparation of the material.

Lipolytic Lipase.

Lecithin itself is readily attacked by lipase. Bokay [1877] showed that lecithin is quickly split by pancreatic lipase into glycerophosphoric acid, a base (which he termed neurin) and fatty acids. The action of ricinus lipase is similar [Schumoff-Simanowski and Sieber, 1906]. Paul Mayer [1906] succeeded in obtaining inactive racemic lecithin by heating ordinary dextro-lecithin with alcohol in a sealed tube for from five to six hours at 90 to 100°. On treating the inactive product with steapsin he obtained a lævo-rotatory solution, the dextro-form being broken down by the enzyme.

Coagulation of Blood.

Extracts of the tissues are known to be capable of influencing the formation of the fibrin ferment, thrombin. It has been customary to regard three factors as essential to the formation of this ferment, i.e. (1) an antecedent substance prothrombin derived from the blood plates, (2) the calcium salts normally present in the plasma, and (3) an activating substance thrombokinase, which may be furnished by the platelets or leucocytes, or indeed may be obtained from the tissues generally. Bordet and Delange [1913] found that lipins, like tissue juices, induced the development of large quantities of thrombin in serum, a result which Rumpf [1913] failed to confirm. Howell [1912] carried out experiments on the thromboplastic action of tissue extracts and arrived at the conclusion that their action was due, not to the presence of an activating substance, but to the fact that they contained a substance capable of neutralising antithrombin, and he attempted to identify this substance as a phosphatide related to kephalin. Jay McLean [1916] tested the action of kephalin purified as far as possible, and found it very active in producing coagulation of a mixture of fresh serum and oxalate plasma.

These observations are not altogether in accordance with the results of other observers; thus Rumpf [1913] found that lipin emulsions prepared from ox brain were *far less* active than tissue extracts in hastening coagulation, and failed to confirm the statement of Bordet quoted above; on the other hand, the same observer found that serum deprived of its lipins by extraction with petroleum would only coagulate after lipins had been again added to it. On the whole it seems probable that lipins do play some part in the coagulation of the blood, but it does not seem certain that it is the lipins which constitute the thromboplastic substance of the tissues.

Tait and Hewitt [1914] examined the lipin content of vascular endothelium with the object of correlating it with the power the endothelium possesses of preventing the coagulation of blood, but they came to the conclusion that the percentage of lipin (8·41 per cent. of the endothelium extract) was not large enough to warrant such a conclusion. Tait and Campbell [1914] made a similar investigation on the lipin content of peritoneal endothelium.

Conclusion.

A perusal of the results of the work undertaken to elucidate the biological significance of the lipins, justifies the assertion that so far the special part played by the lipins in the activity of the organism, is entirely unknown to us. Certain of the results recorded above may perhaps lead to work which will throw some light on the biological functions of these substances. At present, however, we can only await the further development of the subject.

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